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THE LOCALISING ACTION OF FIBRINOUS EXUDATES.

T. D. DAY.

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Received for publication March 18, 1954.

MENKIN (1940) has attached great importance to the part played by fibrin in the localisation of tissue damage. He considers it to act as a mechanical barrier in lymphatics and tissue spaces. The purpose of the present paper is to describe another and quite different way in which fibrin formation may lead to localisation.

MATERIALS AND METHODS.

Although in general it was found convenient to use mouse connective tissue, the principal phenomena described were reproduced in human material. Human connective tissue was obtained from routine autopsies. The caecum, ascending colon and root of the mesentery were gently retracted from right to left and the lamellae of connective tissue thus exposed dissected away intact. Pieces were chosen which were as thin and free of fat as possible. They were placed at once in physiological saline. Mouse connective tissue was obtained as follows: After killing, a median incision was made through the skin of the trunk and extended at each extremity along the ventral aspect of the limbs on one side. The flap thus outlined was gently pulled away from the trunk and pinned on a cork mat. The exposed surface was at once moistened with saline. It is covered by a thin transparent layer of connective tissue, freely movable over the dermis. For the present purpose the entire layer was not removed but only as little of it as could be dissected away in continuity without tearing or undue stretching. Thick pieces make perfusion experiments unduly long.

After excision, the lamella of connective tissue was transferred to the side of a moistened glass tube of 0·65 cm. external diameter and of ordinary thickness and gently spread out to its full extent. It was then possible to draw it over the end of the tube as an occluding membrane. With slight further manipulation it was ensured that the portion actually occluding the tube was as thin and transparent as possible. With the tube held in a clamp the membrane was fixed with a tight rubber ring. It was of the greatest importance that the membrane should be submitted to no further tension whatever during this manoeuvre. Finally the tube was removed from the clamp and re-fixed with the membrane immersed in the appropriate fluid.

Rate of flow through the membrane was measured by the time taken for the meniscus of fluid in the tube to pass between two marks approximately 1 mm. apart and representing a measured volume of about 18 μl. In the experimental conditions described, variations in the time taken for the meniscus to pass between the marks provide a direct measure of changes in the permeability of the membrane and in Fig. 1 and 2 the stopwatch readings are plotted unaltered. Although this results in an inverse representation of rate of flow it was not considered desirable to convert the readings into true velocities since the precise volume of fluid flowing is irrelevant. Furthermore any statement of change in rate of flow in terms

of very small volumes passing in unit time would imply a degree of accuracy in measurement of volume which the method neither possesses nor requires.

These marks were placed approximately 4 cm. above the level of the fluid in which the membrane was immersed. When filled up to the marks with the membrane *in situ* the tube contained slightly over 1 ml. of fluid. By suitable adjustment of the rubber ring it was ensured that the membrane was always sufficiently relaxed to allow it to bulge downwards considerably when supporting a column of fluid 4 cm. high.

The materials used were as follows :

Plasma.—Fresh citrated human plasma as used for therapeutic infusion.
Thrombin.—" Thrombin topical " of bovine origin (Parke Davis & Co.).
Streptokinase.—Burroughs Wellcome & Co.
Hyaluronidase.—" Hyalase," Bengers' Ltd.
Dextran.—Special sample of very large molecular size provided by Dr. F. Fletcher of Bengers' Ltd.
Latex spheres as used for the calibration of electron microscopes.
Citrate solution.—A 1 per cent w/v concentration of sod. citrate.

RESULTS.

The Comparative Effects of Serum, Plasma and Plasma in Process of Clotting on Connective Tissue Permeability; the Reversal of these Effects by Streptokinase.

Sixty N.I.H. units of thrombin were added to 20 ml. of fresh plasma. After 2 hr. at room temperature the resulting clot and fluid expressed from it were allowed to drain through filter-paper. The serum was collected and diluted 5-fold with citrate solution. Another portion of the same sample of plasma was filtered and similarly diluted. A membrane of mouse connective tissue was prepared in the way described and immersed in citrate solution. The membrane was perfused successively with citrate, dilute serum, citrate, dilute plasma and again citrate, each fluid for a few minutes. The rates of flow recorded in Fig. 1 show slight slowing when serum and plasma were perfused, that due to plasma being somewhat greater and more permanent.

Thirty units of thrombin were added to a further 40 ml. of filtered and diluted plasma in a 50 ml. measuring cylinder. The cylinder was slowly inverted at intervals of 2–3 sec. to prevent total coagulation. After 4 min. some of this mixture was perfused through the membrane. Such shreds of clot as formed in the tube were periodically removed. The process of slow agitation was maintained throughout the period of perfusion of this clotting mixture. (After cessation of agitation the fluid in the cylinder clotted completely within 10 min.).

It was observed (Fig. 1) that the passage through the membrane of plasma in the process of clotting decelerated flow far more than either plasma or the serum derived from that plasma after the clotting process was complete.

Citrate solution was substituted for the clotting mixture and the deceleration ended, the flow now being constant at a much slower rate than before. Finally, 50 Christensen units of streptokinase were introduced into the tube immediately above the membrane. Acceleration of flow took place so that in a few minutes citrate solution passed through the membrane at the same rate as at the beginning of the experiment.

Dependence of Slowing on the Continuance of the Clotting Process.

A connective tissue membrane was prepared and immersed in citrate solution as before. Perfusion was begun with plasma diluted two-fold with citrate solution. After a few minutes a strong dose of thrombin (30 units) was added and

well mixed with the plasma in the tube. The contents of the tube clotted solid within 2 min. Rate of flow through both clot and membrane were measured by the addition of small quantities of citrate solution above the clot.

With the onset of clotting there was an almost immediate deceleration of the rate of flow after which the rate became steady (Fig. 2A). After a few minutes most of the clot was removed and the tube washed out well with citrate solution so as to remove any remaining thrombin. Perfusion with plasma was recommenced and maintained for some minutes without further change in rate of flow. A very small dose of thrombin (approximately 2 units)

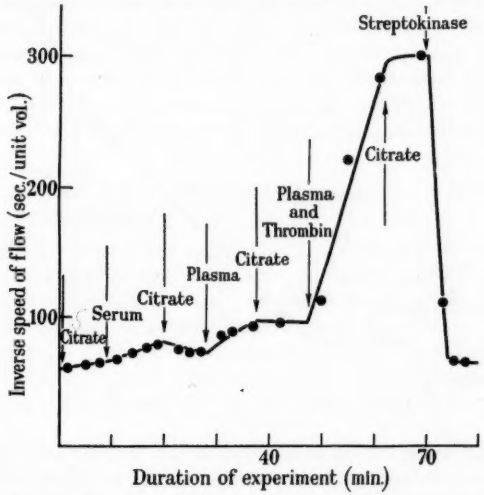

FIG. 1.—Record of changes in rate of flow through a membrane of connective tissue during perfusion with serum, plasma and with a mixture of plasma and thrombin. Reversal of these changes by streptokinase.

was then mixed with the plasma in the tube and perfusion with plasma was continued. No clots were visible in the tube during this period of perfusion but there was a steady deceleration of flow (Fig. 2B). Finally, some 40 min. after the addition of the weak dose of thrombin, a further strong dose was added with precisely the same result as at first.

In the previous experiment clotting was delayed by mechanical agitation. Here, delay was achieved by dilution of the clotting agent. Both experiments point to the same conclusion: that the passage through connective tissue of plasma in which clotting is in progress leads to far greater reduction in permeability than does perfusion with either unclotted plasma or with the serum which remains when clotting is complete.

Dependence of Slowing on the Establishment of Flow.

A tube with membrane attached was immersed to a depth of 2 cm. in 30 ml. of plasma in a small beaker. The tube was filled with plasma to the level of the fluid outside it. Citrate solution was then added up to the timing marks 4 cm.

above this level and the time taken for fluid to pass between them was recorded (41 sec.). The tube was then emptied and fresh plasma added up to the level of the plasma in the beaker. Forty units of thrombin were mixed with the plasma inside the tube and 100 units with that in the beaker. Clotting began in < 1 min. with the result that the membrane became embedded in and permeated by a solid coagulum. After 40 min. citrate solution was again added to the tube and the time taken for the meniscus to pass between the marks was found to be 52 sec. Plasma to which thrombin had been added to the proportion of 30 units/10 ml.

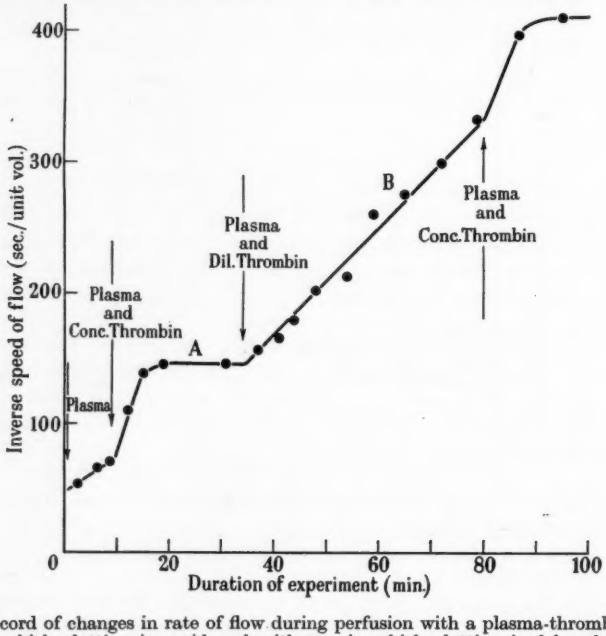

FIG. 2.—Record of changes in rate of flow during perfusion with a plasma-thrombin mixture in which clotting is rapid and with one in which clotting is delayed.

and prevented from solidifying by gentle shaking was substituted for citrate solution. Clots which appeared in the tube were removed as they formed and perfusion was maintained for 10 min. The plasma and such clots as remained were removed from the tube and replaced by citrate solution. The time taken for fluid to pass between the marks was now 202 sec.

This experiment shows that in the absence of flow across it, the formation of a dense clot both within and around a connective tissue membrane results in only very slight diminution of permeability.

Size of Particles Involved in the Slowing Process.

A slightly opaque suspension in citrate solution of latex spheres of a uniform diameter of $0{\cdot}25\mu$ was perfused through a connective tissue membrane for 40 min. without any slowing in rate of flow. The effluent was examined from time

to time and found to be completely clear and transparent. A 0·1 per cent w/v solution of dextran of high molecular weight (> 1,000,000), freshly prepared and filtered through a double thickness Whatman No. 1 filter-paper, was then perfused. There was immediate and marked slowing of flow so that within 10 min. the time taken for fluid to pass between the marks had increased from 42 to 231 sec.

It would seem that particles of a diameter of 0·25μ are unable to pass through the tissue. On the other hand their perfusion in reasonable dilution does not cause slowing of flow. However, a solution of the macromolecular substance dextran of far smaller w/v concentration has a very considerable slowing effect. Previous work (Day, 1952) suggested that the slowing of flow which accompanied perfusion with very large molecules was due to their impaction in holes in the tissue of a similar order of size. It would seem likely that the incapacity of latex spheres to slow the rate of flow is due to their being too big to fit such holes. This experiment suggests that particles of macromolecular size are formed during the clotting process and it is to these that the observed slowing of flow is due.

Comparison of the Action of Streptokinase with that of Hyaluronidase.

As previously described (Day, 1950; 1952), the action of hyaluronidase on a connective tissue membrane is to increase its permeability very greatly. The initial low permeability is restored by the perfusion of the membrane with a variety of macromolecular substances. In the present work it was proved that a plasma-thrombin clotting mixture was similarly capable of counteracting the effect of hyaluronidase. Thus in one experiment the time taken for saline to pass between the marks was decreased from 37 to 2 sec. by hyaluronidase. A clotting mixture was perfused with the result that in 4 min. the time taken was 41 sec., and after 40 min. 430 sec.

At the concentration used in these experiments, the streptokinase preparation increased the permeability of fresh connective tissue to almost the same extent as hyaluronidase. But in their action upon a membrane which had been rendered less permeable by perfusion with a clotting mixture of plasma and thrombin, the two preparations presented a marked contrast. Thus passage of a clotting mixture through a membrane resulted in an increase in the time taken to pass between the standard marks of from 28 to 228 sec. Addition of 50 units of "Hyalase" was followed within 6 min. by reduction in the time taken to 120 sec. with no further alteration. Finally 20 Christensen units of streptokinase were added with restoration within 10 min. of the original rapid rate of flow.

The streptokinase preparation was able to dissolve fibrin clots which the hyaluronidase preparation could of course not do. It is probable that the complete reversal by streptokinase of the slowing effect induced by perfusion with a plasma-thrombin mixture is referable to this property. It may also be assumed that the partial reversal brought about by hyaluronidase is due to the action of this enzyme on hyaluronic acid normally present in the tissue. It would appear from its effect on fresh connective tissue that apart from its action on fibrin the streptokinase preparation contains a permeability factor.

DISCUSSION.

These experiments show that the process of fibrin clotting leads to considerable diminution of the permeability of connective tissue provided that clotting is

actually in progress and that flow through the tissue is established. This result is in accordance with a concept of the fine structure of connective tissue suggested by previous work (Day, 1952). Connective tissue was considered there to consist essentially of a protein network having meshes of macromolecular dimensions. Slowing of flow was shown to take place when solutions of macromolecular substances such as starch or dextran were perfused through the tissue and this slowing was regarded as due to blockage of the meshes by macromolecules or macromolecular aggregates. If it be assumed in the present instance that particles are formed during the clotting process of such a size as to be impacted in a similar way, the reason for the ensuing decrease in permeability is clear. It is significant in this connection that Porter and Hawn (1949) observed protofibrils 200–230 Å in diameter in electron micrographs of fibrinogen in the early stages of clotting.

A matter of greater interest perhaps is the bearing of these results on the rôle of fibrin in the acute inflammatory process. The pressure at which these perfusion experiments were performed (4 cm. water) lies within the range of pressures obtaining in the blood capillaries. Moreover the addition of thrombin to citrated plasma is not perhaps a too far-fetched representation of the liberation into a fibrinogen-rich exudate of a clotting agency from the tissues either as the direct result of damage or, as in the case of staphylococcal infection, elaborated by the noxious agent itself. The important change is the transformation of fibrinogen to fibrin and it is not material to the present argument how this change is brought about. The experimental conditions may therefore be regarded as a model of what might be expected to happen in connective tissue when an acute inflammatory exudate develops *in vivo*. The model suggests the existence in connective tissue of a localising mechanism of some subtlety since in favourable circumstances the passage of a fibrinogen-containing exudate through the tissue must automatically decrease its permeability when, as in injury, a clotting principle is liberated.

The kind of permeability discussed above is concerned with the passage of water through a membrane of connective tissue. It remains to consider to what extent changes in this kind of permeability may be related to the localisation of pathological noxae *in vivo*. The experimental membrane may be compared to a zone of connective tissue which immediately surrounds and is compressed by a focus of developing acute inflammatory exudation. On this analogy, it is apparent that diminution in the flow of water through this zone must result in a reduction in the spread of tissue fluid and lymph into the surrounding normal tissue. In this way reduction of permeability of connective tissue to water must result in limitation of the spread of noxious matter from an inflamed area.

SUMMARY.

Perfusion of connective tissue with plasma in which clotting is in actual progress resulted in considerable decrease in permeability. This effect was considered to be due to the formation during the process of clotting of particles of fibrin of a macromolecular order of size, and the subsequent impaction of these in the tissue.

The bearing of this finding on the rôle of fibrin in the acute inflammatory process is discussed.

I wish to thank Dr. J. A. V. Shone, of the Leeds Blood Transfusion Centre, for his most kind co-operation, and Dr. G. C. Turner, of the Department of Bacteriology, Leeds University, for some valuable suggestions as to choice of material.

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THE ABILITY OF PERTUSSIS VACCINE TO PRODUCE IN MICE SPECIFIC IMMUNITY OF A TYPE NOT ASSOCIATED WITH ANTIBODY PRODUCTION.

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Two methods have been suggested for controlling the prophylactic potency of pertussis vaccines. The first, devised by the U.S. National Institutes of Health and the Michigan Department of Health (Kendrick, Updyke and Eldering, 1949; Pittman, 1952), necessitates the immunization of mice with a single inoculation of vaccine and the determination of the degree of immunity by the intracerebral challenge injection of virulent *Haemophilus pertussis*. In the second method (Evans and Perkins, 1953; 1954) tests are made of the ability of two injections of pertussis vaccine to produce specific agglutinin in mice. It is not yet known whether either of these methods is correlated with the ability of pertussis vaccines to protect children against whooping cough and it will be necessary to await the results of the Medical Research Council field trials (Report, 1951) before the value of these methods can be assessed.

Although we suggested (Evans and Perkins, 1953), from the results of a few preliminary tests, that there was a rough parallelism between the ability of vaccines to protect mice against intracerebral infection and their power to produce agglutinin, this did not imply that the agglutinogen of *H. pertussis* was responsible for inducing protection. In fact, there is no evidence at present that the immunity produced in mice, as a result of a single injection of vaccine, is due to the production of antibodies to any of the known antigens of *H. pertussis*. We have therefore investigated the relationship between the development of immunity in mice following the injection of pertussis vaccine and the production of specific antibodies.

MATERIALS AND METHODS.

Vaccines.—Three pertussis vaccines designated V12, V16 and V17, prepared for the Medical Research Council's field trials, were used. Vaccines V12 and V16 were whole bacterial suspensions containing 20×10^9 organisms/ml. Vaccine V17 was a bacterial extract prepared by Pillemer, Blum and Lepow (1954) and referred to by them as SPA. The reference vaccine YPS which we prepared for agglutinin production tests (Evans and Perkins, 1954) was also used; this contained 20×10^9 organisms/ml.

Inoculation of mice.—Female white mice were used. Their average weight on first inoculation was 16 g. The immunizing dose of vaccine was always contained in 0·5 ml. and was given intraperitoneally. Vaccines V12, V16 and YPS were diluted 1/4 in saline, so that the immunizing dose contained $2{\cdot}5 \times 10^9$ organisms. Vaccine V17 was also diluted 1/4 in saline for it had been shown that this vaccine was as potent as the whole bacterial vaccines in protecting mice against intracerebral infection.

Determination of immunity.—Tests for immunity were made by challenging mice with an intracerebral injection of a suspension of virulent *H. pertussis* according to the method described by Kendrick, Eldering, Dixon and Misner (1947), using the challenge strain of

H. pertussis, No. 18/323, which they recommended. The challenge dose in all tests contained between 75 and 125 LD_{50} calculated by the method of Reed and Muench (1938). The mice were observed for 10 days after challenge during which time the number of deaths and times to death were recorded.

In vitro *tests for antibody*.—Sera, obtained from immunized mice by the method we have described (Evans and Perkins, 1954), were titrated for agglutinin and complement-fixing antibody.

The agglutinin titration followed the method we used previously, employing as antigen a living suspension of *H. pertussis* containing 30×10^9 organisms/ml. Mixtures of antigen and serum dilutions were incubated for 1 hr. at 37° and left at room temperature for 18 hr. before reading. A control antiserum (titre 64,000) was always included in the tests.

Complement-fixation titrations were made with a suspension of *H. pertussis*, killed by 1/10,000 thiomersalate, containing $6{\cdot}5 \times 10^9$ organisms/ml., 3 M.H.D. of complement and a 2 per cent suspension of sheep R.B.C. sensitized with 3 M.H.D. of haemolysin. Fixation was for 1 hr. at 37° and after adding the haemolytic system there was further incubation at 37° for ½ hr. A control pertussis antiserum (titre 128) was always included in the tests.

In vivo *tests for protective antibody*.—In testing for protective antibody groups of normal mice were given intraperitoneally a dose of 1 ml. of pooled sera taken from a group of immunized mice. About 2 hr. later the mice were challenged intracerebrally with graded doses of a living suspension of *H. pertussis*. Control tests were also made with groups of mice which received normal mouse serum and groups which received no serum. The mice were observed for 10 days after challenge and the number of deaths and times to death recorded.

EXPERIMENTAL AND RESULTS.

Development of Immunity.

Tests for the development of immunity after injection were made with each of the 4 vaccines. In a single test 5 groups of mice, 10 to 15 mice in each group, were used. Each group was inoculated at a different time over a period of 10 days and all the groups were challenged intracerebrally on the same day with the same challenge dose of living *H. pertussis* containing between 75 and 125 LD_{50}. The times at which the vaccine was injected were spaced so that the challenge dose was given at 5 hr., 1, 3, 6 and 10 days after injection to each of the 5 groups of mice respectively. The challenge suspension in each test was titrated in normal mice to determine the number of LD_{50} contained in the challenge dose.

In all, 11 separate tests were made, 2 tests with each of the whole bacterial vaccines V12, V16 and YPS and 5 tests with the bacterial-extract vaccine V17. The results of each of the 11 tests were very similar and show that with each vaccine there was a rapid development of immunity to intracerebral infection (Table I). The combined results, which are similar to those with each vaccine, show that at 5 hr. after injection of vaccine 31 per cent of mice were immune to infection, at 1 day 52 per cent, at 3 days 70 per cent and at 6 and 10 days almost all were immune. Of the 165 control mice, which did not receive vaccine, only 1 survived the challenge injection.

Experiments were also made to see if vaccine given after the challenge dose was able to induce immunity. Results showed that mice which received vaccine 1 day after challenge were not immune and were as susceptible to a challenge dose of approximately 100 LD_{50} as mice which received no vaccine.

In Vitro *Tests for Circulating Antibody.*

In vitro tests for circulating antibody were made with vaccines V16, V17 and YPS. Each was injected into 3 groups of mice, about 10 mice per group, and sera taken 1, 3 and 14 days later from each of the 3 groups respectively. The pooled

TABLE I.—*Development of Specific Immunity in Mice after a Single Dose of Pertussis Vaccine.*

Vaccine.	No. of tests.	Proportion (and percentage) of mice surviving challenge. Interval between immunizing dose and challenge dose: 5 hr.	1 day.	3 days.	6 days.	10 days.	Challenge dose only.
YPS	2	16/27 (59)	22/27 (82)	17/27 (63)	26/27 (96)	25/25 (100)	0/30 (0)
V12	2	8/26 (31)	18/27 (67)	21/26 (81)	22/25 (88)	25/25 (100)	0/30 (0)
V16	2	7/27 (26)	7/27 (26)	21/26 (81)	25/26 (96)	22/27 (82)	0/30 (0)
V17	5	14/67 (21)	30/67 (45)	35/55 (64)	58/65 (89)	61/65 (94)	1/75 (1)
Total	11	45/147 (31)	77/148 (52)	94/134 (70)	131/143 (92)	133/142 (93)	1/165 (0·6)

The challenge doses contained between 75 and 125 LD_{50}.

sera from each group were titrated for agglutinin and complement-fixing antibody. Agglutinin titrations were also made with the individual sera from each mouse.

Table II shows that with each vaccine a single inoculation produced a poor antibody response. There was no detectable complement-fixing antibody in any of the sera and agglutinin was detected only in low titre in sera taken on the fourteenth day after inoculation. The results of the agglutinin titrations with

TABLE II.—In vitro *Tests for Specific Antibody in Sera of Mice Immunized with a Single Dose of Pertussis Vaccine.*

Vaccine.	No. of days after inoculation when sera taken.	No. of sera.	Antibody titres of pooled sera. Agglutinin.	Complement-fixing antibody.
YPS	1	10	<10	<2
	3	10	<10	<2
	14	12	10	<2
V16	1	10	<10	<2
	3	10	<10	<2
	14	12	20	<2
V17	1	10	<10	<2
	3	10	<10	<2
	14	11	10	<2

the individual sera also indicated that each vaccine produced a poor agglutinin response. Of the 32 sera from mice inoculated with YPS, 1 taken at 3 days and 1 taken at 14 days after inoculation had a titre of 40 and all the rest had titres of < 40; of the 32 sera from mice inoculated with V16, 1 serum taken at 14 days had a titre of 80, 3 others also taken at 14 days each had a titre of 40 and all the rest had titres of <40; of the 31 sera from mice inoculated with V17, all had titres of <40.

Test for Protective Antibody.

Tests for protective antibody were made with sera of mice inoculated with V16 and V17. With each vaccine 150 mice were inoculated, and 3 days later, at a time when an average of 70 per cent of mice were immune to the intracerebral challenge injection of at least 100 LD_{50}, all the mice were bled and their sera pooled. The protective property of the pooled sera was tested in 5 groups each containing 10 mice. The mice were given 1 ml. of serum intraperitoneally and about 2 hr. later challenged intracerebrally with living *H. pertussis*, using doses ranging from 100 LD_{50} to less than 1 LD_{50}. Five groups of mice which received about 2 hr. previously 1 ml. of pooled serum from normal mice and 5 groups which received no serum were also challenged with the same doses.

TABLE III.—*Tests for Protective Antibody in Sera of Mice Immunized with a Single Dose of Pertussis Vaccine.*

Serum given 2 hr. before challenge.	Proportion of mice surviving after challenge with the following doses (LD_{50}).					Proportion of totals.
	100.	20.	4.	0·8.	0·16.	
None	1/10	0/10	2/10	5/10	9/10	17/50
Normal mouse serum .	0/10	1/10	1/10	6/9	8/10	16/49
Anti V16 serum . .	0/10	0/10	1/10	7/10	8/10	16/50
Anti V17 serum . .	0/10	1/10	0/10	8/11	6/10	15/51

Anti V16 and anti V17 sera were obtained from 2 groups of mice 3 days after giving a single dose of V16 and V17 to each group respectively.

The results of these tests (Table III) show that serum from immunized mice was unable to protect mice passively against even small infecting doses of *H. pertussis*. The numbers of deaths were strikingly similar in all sets of mice as was the time taken to die. It may be argued that the dose of 1 ml. of serum was too small to detect protective antibody. It is considered, however, that such an argument does not apply in view of the fact that the serum was taken from mice at a time when they showed a high level of immunity to the intracerebral challenge injection of 100 LD_{50} of *H. pertussis* and that 1 ml. of serum was unable to protect passively against a challenge injection of less than 1 LD_{50}.

Tests for Antibody in Sera of Mice Inoculated with Vaccine and then Challenged.

The above tests show that a single injection of vaccine produced in mice a high degree of immunity to intracerebral infection and yet produced little or no detectable circulating antibody at the time of challenge. The possibility was considered, however, that circulating antibody might be produced as a result of the challenge injection acting as a secondary antigenic stimulus. This possibility was investigated using vaccines V16, V17 and YPS.

A group of 130 mice was inoculated with V16, challenged intracerebrally 3 days later with 120 LD_{50} and 6 days after challenge, at a time when non-immune mice usually began to die, the mice were bled. The sera from all the mice were pooled and tested for the presence of protective antibody using normal mice as before. The results (Table IV) show that mice which received the pooled serum were as susceptible to intracerebral infection with *H. pertussis* as mice which received no serum.

TABLE IV.—*Tests for Protective Antibody in Sera of Mice Immunized with a Single Dose of Pertussis Vaccine, Challenged 3 Days Later and Bled 6 Days after Challenge.*

Serum given 2 hr. before challenge.	Proportion of mice surviving after challenge with the following doses (LD_{50}). 50.	10.	2.	0·4.	Proportion of totals.
None	0/10	3/10	1/10	8/10	12/40
From mice immunized with V16 and challenged	0/10	1/10	6/10	9/10	16/40

Further tests were made with vaccines V16, V17 and YPS to see if the challenge dose produced an increase in the agglutinin or complement-fixing antibody titres of the sera of immunized mice. Groups of mice were inoculated with vaccine challenged either 3 or 7 days later with between 80 and 120 LD_{50} and bled either 6 or 10 days after challenge. *In vitro* tests for antibody were made with the pooled sera from each group. The results (Table V) show that there was little or no detectable antibody in the sera of any of the groups.

TABLE V.—In vitro *Tests for Specific Antibody in Sera of Mice Immunized with a Single Dose of Pertussis Vaccine, Challenged 3 or 7 Days Later and Bled 6 or 10 Days after Challenge.*

Vaccine.	No. of days after vaccine when challenged.	No. of days after challenge when sera taken.	No. of sera.	Antibody titres of pooled sera. Agglutinin.	Complement-fixing antibody.
V16	3	6	26	<10	<2
V16	3	10	13	10	2
V16	7	10	14	40	2
V17	3	6	21	<10	<2
YPS	3	10	13	<10	<2
YPS	7	10	12	<10	<2

The challenge doses contained between 80 and 120 LD_{50}.

These results show that the challenge dose did not act as a secondary antigenic stimulus, since protective antibody, agglutinin and complement-fixing antibody were no more detectable in the sera of the mice which had been given vaccine and then challenged, than in the sera of those which had received vaccine alone.

Antibody Response after a Second Injection of Vaccine.

Tests were made with vaccines V12, V16 and V17 to determine the extent of the antibody response resulting from a second injection of vaccine. In testing each vaccine a group of about 20 mice was given 2 equal doses of vaccine at an interval of 14 days. Each dose was the same as that used in the single inoculation tests. Sera were taken at 6 days after the second dose from about half the mice and at 12 days from the remainder. Agglutinin titrations were made on the individual sera, and complement-fixation titrations on the pooled sera. The results of these tests are shown in Table VI and the Figure. A good secondary response of both agglutinin and complement-fixing antibody was produced by the whole bacterial vaccines V12 and V16; at 6 days average agglutinin titres were 500 and 1350 and complement-fixation titres 32 and 32; at 12 days even

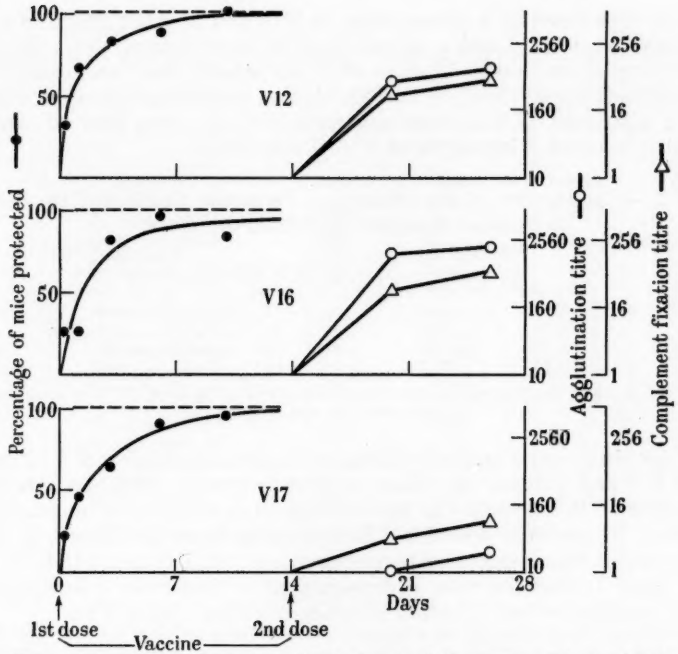

FIGURE.—The relationship between the development of immunity and circulating antibodies in mice inoculated with pertussis vaccines V12, V16 and V17. (Logarithm scales used for antibody titres.)

slightly higher titres were obtained. On the other hand the response produced by the second injection of the bacterial extract vaccine V17 was comparatively poor; average titres for agglutinin were 10 and 20 and for complement-fixing antibody 4 and 8 at 6 and 12 days respectively.

TABLE VI.—In vitro *Tests for Specific Antibody in Sera of Mice Immunized with Two Doses of Pertussis Vaccine Separated by an Interval of* 14 *days.*

Vaccine.	No. of days after second inoculation when sera taken.	No. of sera.	Antibody titres. Agglutinin (single sera, (geom. mean).	Antibody titres. Complement-fixing antibody (pooled sera).
V12	6	14	500	32
	12	13	700	64
V16	6	8	1350	32
	12	11	1700	64
V17	6	9	10	4
	12	12	20	8

The difference in the ability of the 2 types of vaccine to produce antibody was also shown by tests in which 1 group of mice was given a first dose of V17

followed 14 days later by a second dose of V16 and another group of mice was given a first dose of V16 and a second dose 14 days later of V17. The average agglutinin titre of sera taken 12 days after the second dose from each of these 2 groups, although higher than the average titre of sera from a group of mice which received 2 injections of V17, was substantially lower than that of sera from a group which received 2 injections of V16 (Table VII).

TABLE VII.—*Comparison of the Ability of Pertussis Vaccines V16 and V17 to Produce Specific Agglutinin in Mice.*

Vaccine.		No. of sera tested.	Agglutinin titre (single sera, geom. mean).
First dose.	Second dose.		
V16	V16	11	1700
V16	V17	14	230
V17	V16	12	270
V17	V17	12	20

The two doses of vaccine were separated by an interval of 14 days and sera were taken 12 days after the second dose.

Although there was a marked difference between the ability of V17 on the one hand and V12 and V16 on the other to produce specific antibody, this difference was not reflected in the ability of the vaccines to produce early immunity against intracerebral *H. pertussis* infection. It is evident from the Figure, in which we have also plotted the immune-response curves of V12, V16 and V17 from the data given in Table I, that the rate of development of immunity following injection of V17 was similar to that following injection of either V12 or V16.

Specificity of Pertussis Vaccine to Produce Immunity to Infection.

Experiments were made to determine whether the production of early immunity to intracerebral infection with *H. pertussis* was specific for pertussis vaccine. Three groups of mice were inoculated intraperitoneally with 0·5 ml. of a 1/4 dilution in saline of each of the following antigenic preparations. Vaccines V16 and YPS; suspensions of *Haemophilus bronchisepticus, Haemophilus parapertussis, Bacterium coli, Chromobacterium prodigiosum,* (each suspension was killed at 56° for ½ hr. and contained 20×10^9 organisms/ml.); diphtheria P.T.A.P.; tetanus toxoid; a 24 per cent solution of dextran; normal ox serum. In each test, each of the 3 groups of mice was inoculated at a different time over a period of 7 days and all were challenged intracerebrally with 80 to 100 LD_{50} on the same day. The times of injection of vaccine were spaced so that the challenge dose was given at 1, 3 and 7 days after inoculation with each of the 3 groups respectively. The challenge suspension was always titrated in normal mice to determine the number of LD_{50} contained in the challenge dose.

These tests show that definite early immunity was produced only by the pertussis vaccines V16 and YPS (Table VIII). There was some evidence in one test that the suspension of *H. parapertussis* produced a slight degree of immunity in the mice challenged at 7 days although those challenged at either 1 or 3 days showed no immunity. All the other antigenic preparations produced no immunity.

We also included in this test a group of mice inoculated with vaccine YPS which had been heated at 100° for 15 min.; this heated vaccine produced no immunity to infection after either 1, 3 or 7 days.

TABLE VIII.—*The Specificity of Pertussis Vaccine to Produce Immunity in Mice.*

Test No.	Antigen.	Proportion of mice surviving challenge. Interval between antigen and challenge dose. 1 day.	3 days.	7 days.	Challenge dose only.
1	*H. pertussis* YPS	9/15	13/15	12/15	
	H. pertussis V16	5/15	14/15	13/14	
	H. parapertussis	1/15	2/13	5/15	0/15
	Bact. coli	1/10	0/13	1/15	
	Diphtheria P.T.A.P.	0/15	1/14	0/15	
2	*H. pertussis* YPS	7/13	13/13	13/15	
	H. bronchisepticus	2/13	1/13	1/15	
	Chr. prodigiosum	1/13	0/12	0/15	0/15
	Tetanus toxoid	1/13	2/13	0/15	
	Ox serum	1/14	1/13	0/15	
3	*H. pertussis* YPS	7/13	10/12	12/13	
	H. parapertussis	1/13	0/13	1/13	
	H. bronchisepticus	0/12	2/12	1/13	0/15
	Dextran	0/13	1/13	0/13	
	Heated YPS	0/13	0/13	0/13	

The challenge doses contained between 80 and 100 LD_{50}.

CONCLUSIONS.

These experiments have shown that a single intraperitoneal injection of pertussis vaccine was able to produce in mice, within a few hours after inoculation, a substantial degree of immunity to an intracerebral injection of virulent *H. pertussis*. This effect appeared to be specific for pertussis vaccine. It was not produced by vaccine heated at 100° for 15 min.

When the degree of immunity was determined at different times after inoculation, the immune-response curve obtained was steep, it had no lag-period and was quite unlike the response which is associated with antibody production.

It was also shown that immunity occurred at a time when circulating specific antibodies were not detectable. Even 3 days after injection of vaccine, when immunity to infection was at a high level, no protective antibody or complement-fixing antibody and only a slight trace of agglutinin was detectable in the sera.

Furthermore, it was shown that a challenge injection, given to mice 3 or 7 days after injection of vaccine, did not act as a secondary antigenic stimulus. There was no detectable protective antibody or any perceptible increase in agglutinin or complement-fixing antibody in the sera of immunized mice 6 to 10 days after challenge, a time when non-immunized challenged mice usually died.

The ability of a vaccine to produce early immunity was not associated with its ability to produce specific agglutinin or complement-fixing antibody. A bacterial extract vaccine which gave a poor antibody response after 2 injections was able to induce early immunity as effectively as whole bacterial vaccines which produced a rapid and high secondary antibody response.

These results strongly suggest that the early immunity produced in mice, as a result of a single injection of pertussis vaccine, is not due to the production of specific antibody and is of a type different from that associated with antibody production. The mechanism of this early immunity and whether it is of the

" interference " or " sparing " type met with in the field of virology are being investigated. In this respect it is interesting to note that Dalldorf, Cohen and Coffey (1947) have shown that vaccinia virus induces resistance in mice to intracerebral infection with *H. pertussis*.

Investigations are also being made to determine whether the immunity resulting from a single injection of vaccine is of long duration and whether the factor responsible for producing early immunity is antigenic and capable of producing protective antibody after repeated injections. There are also the important questions of whether the ability of a vaccine to produce early immunity in mice is related to its prophylactic activity in children and if so, whether the same mechanism which produces immunity in mice is also responsible for producing immunity in children. These questions can only be answered by the results of field investigations.

SUMMARY.

A single intraperitoneal injection of pertussis vaccine has been shown to produce in mice an early immunity to intracerebral infection with *H. pertussis*. The immunity appears to be independent of circulating antibodies.

We wish to thank the Medical Research Council for samples of pertussis vaccines received from the Whooping Cough Immunization Committee and for a grant towards the expenses of this work.

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AN IMPROVED METHOD FOR THE PRODUCTION OF TUBERCLES IN A CHAMBER IN THE RABBIT'S EAR.

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THE transparent rabbit ear chamber, as introduced by Sandison (1924, 1928) and later elaborated by Clark and his co-workers (1930), has been further modified so as to produce standard conditions for the observation of growing tubercles. The principal innovation has been the provision of a hole, normally plugged by a metal pin, through which tubercle bacilli can be introduced. A Perspex (lucite) plug was used for this purpose by Ebert, Ahern and Bloch (1948), but its larger size was often the cause of mechanical trauma to the tissue.

EXPERIMENTAL.

The Chamber.

The chamber was made in Perspex. Its main dimensions were similar to those of the chambers used by Ebert, Florey and Pullinger (1939).

Three modifications were introduced for the present work. In some preliminary experiments with Wells and Wylie (1948–49, unpublished) it was found that the reaction of the tissue to inoculation with tubercle bacilli depended to some extent on the thickness of the tissue, and on the rigidity with which it was anchored within the chamber. A loose tissue, insecurely anchored, was subject to frequent small traumata which confused the picture of the infective process, and was usually the site of a considerable exudation of free fluid.

The diameter of the central table was reduced from 8 to 5 mm. The Perspex buffers, separating table and coverslip, which determined the thickness of the tissue in the chamber, were cut from a ring of Perspex 0·5 mm. wide and 30μ higher than the table, which was stuck round the central table like a collar. Parts of this ring were milled away to allow the new tissue to grow onto the central area (Fig. 1).

The relatively large Perspex plugs previously used to fill the hole in the central table were difficult to remove without causing damage to the young tissue, so a metal pin 0·5–0·6 mm. diameter was substituted. The pin was of such a length that it protruded through the chamber and was in contact with the coverslip. In most experiments the pin was made of silver, but zirconium proved more satisfactory and it is probable that tantalum would be equally good. Stainless steel was found not to be satisfactory. The details of the pin are shown in Fig. 2. The small flange near the outer end was important because it protected the Perspex base of the chamber from being scratched when the protruding end of the pin was manipulated with a pin-vice.

In common with the practice of other workers the use of separate splints and protective shields on the ear was abandoned. The bolts, preferably made of zirconium, are now made sufficiently long to hold thin Perspex covers in place on each side of the chamber during the intervals between examinations. They differ from the plain silver bolts previously used in being detachable from the chambers and in having a flange near the middle. The faces of the flange are made very accurately at right angles to the long axis of the bolt. When the bolt is dropped into a clearance hole in the base of the chamber the flange fits into a recess in the base. A brass locknut on the outside holds the bolt in place. The advantage of this

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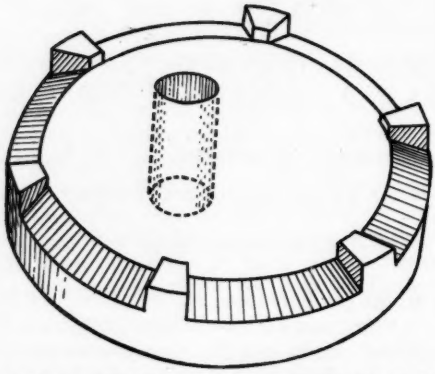


FIG. 1.—Enlarged view of the central table with 6 buffers external to it. The ramps in the Perspex collar from which the buffers are made are well seen at the sides of the table. The hole for the removable pin is also shown.

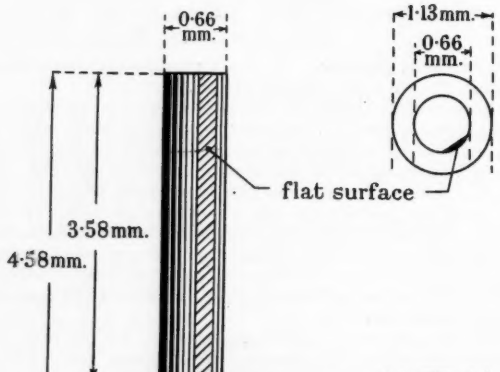


FIG. 2.—Detail of removable pin. The dimensions of a typical pin are given. The length of the shank (3·58 mm. in the illustration) varies from one pin to another and must equal the thickness of the Perspex sheet actually used *plus* the height of the buffers. Below the shank is the flange, which serves the dual purpose of preventing the pin from being pushed in too far and preventing the pin-vice used for handling it from scratching the base of the chamber. The part shown below the flange is gripped by the jaws of the vice. The two end views are shown on the right of the perspective view. Note the flat surface on the shank; the need for this is described in the text.

separate bolt, illustrated in Fig. 3, is that it can be removed for cleaning and can be dry-sterilised independently of the base of the chamber.

The Observation Microscope.

For holding the chamber on the moving stage of the microscope a modification of the ring shown to one of us by Robertson (1951) was used. This is a brass ring with three holes in it which is fixed over the bolts projecting from the base of the chamber by three 10BA brass nuts (Fig. 4*b*).

The rabbit is moved near the microscope and the ear with the chamber and ring is arranged so that the latter fits into a circular hole in the superstage fitting (Fig. 4*a*). It is clamped in position by the screw C.S. The arrangement has the advantage that, should the rabbit become restless, the ear can be released very quickly by a turn of the clamping screw.

Insertion of Chamber.

The method of operation was essentially that described by Ebert *et al.* (1939) but post-operative treatment has been modified.

One of the difficulties associated with long maintenance of chambers is the gradual encroachment of infection due to staphylococci from the edges of the chamber, particularly from the bolt holes. Various local applications were used but the most successful procedure was to administer 200,000 units of penicillin intramuscularly just before operation and to continue this dose daily for the next 10 days. By this means sepsis was almost completely abolished.

A further innovation was the use of an external antiseptic around the chamber after the operation. After several trials 1/1000 acriflavine in a thick solution of gum tragacanth was found to be suitable. This is applied as a thick jelly which soon dries down to a strongly adherent but water-soluble scale. It seals off the danger points round the holes for the bolts.

Inoculation of the Chamber.

The organism used to infect the tissue was the Branch strain of bovine type tubercle bacilli. A history of this strain was given by Mackaness, Smith and Wells (1954). It has been maintained in this laboratory in artificial media with occasional animal passage for 6 years, and has remained highly virulent for rabbits and guinea-pigs. For inoculation into the chamber, the organism was cultured in fluid Dubos-Davis medium for 6 days, a day or two longer than the time required for the appearance of maximal turbidity.

After the tissue in the chamber had organized to the required stage the rabbit was anaesthetized by the intravenous injection of nembutal (30 mg./kg.). The chamber was fixed to the stage of a microscope with the base uppermost so that the projecting stem of the pin could be grasped by a pin-vice (Fig. 5 and 6). This pin-vice was fixed into a brass collar with a standard RMS objective thread on it. It was then inserted in place of the objective of a suitable microscope from which the body tube had been removed. Vertical movements of the pin-vice were controlled by the coarse and fine adjustments which normally actuated the objective head. The table area was illuminated obliquely from above by two spotlights. The pin was placed immediately under the vice by movement of the mechanical stage, aided by observation with two dissecting microscopes set at right angles to each other just above the plane of the stage. In order to observe

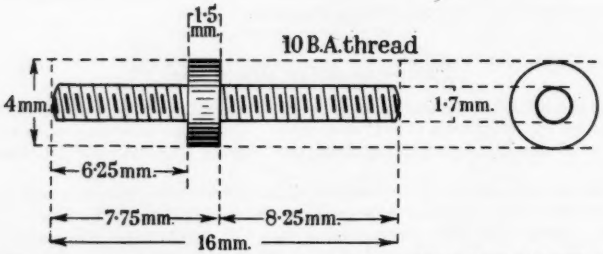


FIG. 3.—Details of the zirconium bolt. It is turned from a single piece of 4 mm. diameter rod.

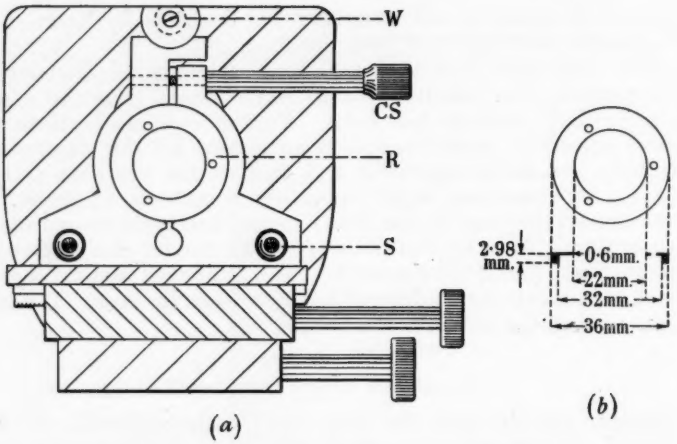
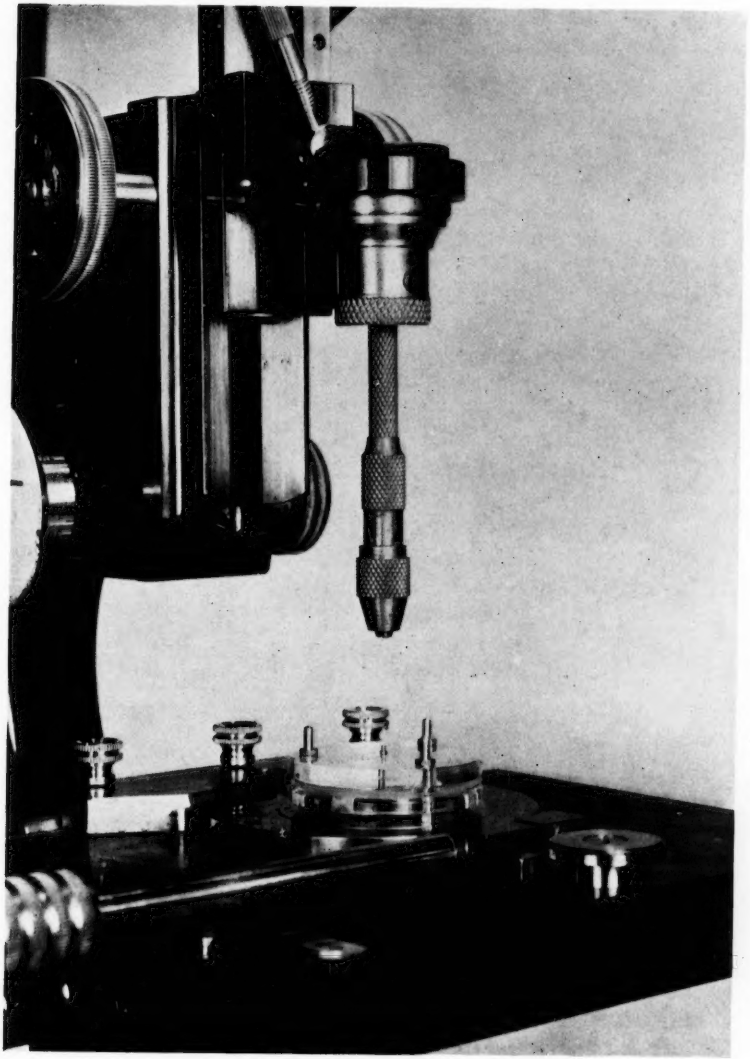


FIG. 4*a*.—The special superstage fitted to the movable stage of a Watson microscope. The stage is shown cross-hatched and the superstage is left plain. The latter is fixed to the cross-movement of the mechanical stage by screws with knurled tops (S). At the far side of the stage a washer (W) over a spacing piece is fixed. The superstage can be moved sideways under this washer, which prevents it from lifting should the rabbit move its head. The central ring (R) is removed by loosening the clamping screw (CS). This ring is slipped over the bolts projecting from the base of the chamber and is secured to the chamber by three 10BA nuts. The assembly of chamber and ring is then placed in the central recess of the superstage, where it finds its own orientation and is then clamped in position by tightening the clamping screw (CS). A similar superstage fitted to a Leitz microscope is shown in Fig. 5.

FIG. 4*b*.—Details of the loose ring (R). Plan and section.

EXPLANATION OF PLATE.

FIG. 5.—A view of a stage of a Leitz microscope with superstage and a chamber in position, *base upwards*, to illustrate the technique of inoculating the chamber. In practice, of course, this is only done after the chamber has been inserted into a rabbit's ear, but the presence of the rabbit in the photograph would obscure the details of the apparatus. For sequence of manipulations see Fig. 6.



Sanders, Dodson and Florey.

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any changes in the tissue during withdrawal of the pin the chamber was watched through a telescope set in front of a prism placed on the substage of the microscope.

The projecting stem of the pin and adjacent area of the chamber base were covered for 5 min. with 1/1000 alcoholic acriflavine, which was then wiped away with absolute alcohol. The pin was grasped by the vice and withdrawn. A

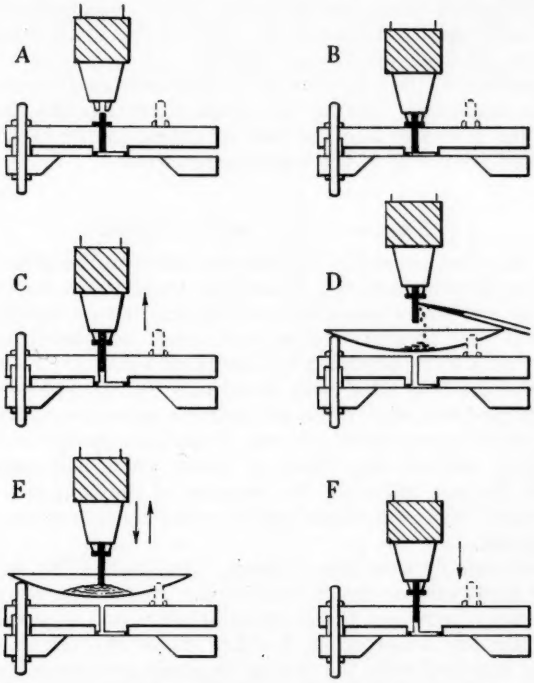

FIG. 6.—The sequence of manipulations for removing the pin and inoculating the tissue with tubercle bacilli. A. The pin-vice with open jaws is lowered over the outer end of the pin. B. The jaws are closed by rotating the knurled finger-grip of the pin-vice. C. The pin is raised by using the fine focusing adjustment of the microscope. D. A watch-glass is rested on the bolts and the débris removed from the pin. E. A sterile watch-glass is then substituted for the first one. It holds a drop of a suspension of tubercle bacilli. The pin is lowered into this drop and then raised again. F. The pin is re-inserted into the chamber. Its tip carries tubercle bacilli to the tissue covering the table.

primary objective during this manoeuvre was to avoid damage to the young tissue growing around the inner end of the pin. In early experiments the pin, which was round, was pulled out as slowly as possible, but in later experiments, when a pin with a very small flat surface along one side to allow ingress of air was used, it was found that less bleeding occurred if the pin was extracted fairly rapidly (see Fig. 2).

The pin was racked well above the stage, and the thin film of dried serum which frequently coated the shank of the pin was scraped away with a sterile

toothpick into a watch-glass placed below. Another watch-glass containing a drop of the Dubos-Davis culture was then placed under the pin, and the end of the pin was lowered into the culture and then withdrawn (Fig. 6).

The drop of culture on the pin was observed through one of the dissecting microscopes until it dried. The pin was then lowered into its hole until the flange was about 0·5 mm. above the base of the chamber. A drop of water-soluble glue was then run around under the flange and the pin was pushed home. Excess glue was wiped away with a moist swab. The centre of the chamber base was again flooded with alcoholic acriflavine solution.

During re-insertion of the pin a primary object was again to avoid damage to the tissue in the chamber. During the whole operation the tissue was clearly visible through the telescope and the rate of extraction or insertion of the pin could be controlled according to the vascular reactions.

Trauma Caused by Inoculation.

The amount of injury caused to the delicate tissue on the table by these operations depended on several factors. Sometimes there was a minute gap between the top of the pin and the coverslip into which one or two capillaries had grown. When such vessels were present, or if a blood vessel completely encircled the pin, the extraction was always attended by haemorrhage. At the most, the lower one-quarter of the pin-hole filled with blood and during replacement of the pin this blood was pushed out with a few air bubbles onto the surface of the tissue. Even when no blood was present air was sometimes pushed into the chamber. In addition, débris, derived from blood or serum which had seeped between the pin and walls of the hole following the insertion of the chamber, and had subsequently dried there, was sometimes pushed onto the tissue on the table when the pin was replaced.

Injured vessels usually went into " stasis " for about 12 hr. and if the trauma was sufficiently great one or two thrombosed. Blood and air pushed into the chamber temporarily increased the internal pressure and stopped the circulation near the pin. Air was absorbed in 3–4 hr. Since the circulation returned in about 6 hr. this was probably the period required to restore the normal extravascular pressure. Fresh blood usually took 3–4 days to be absorbed but dry débris caused a more protracted reaction. When this was realised great care was taken to clean the pin before reinserting it. The débris was phagocytosed by macrophages which, thus labelled, could often be seen to remain in the area indefinitely.

Usually the amount of damage the tissue sustained was very small. Generally the chamber was completely restored to normal before tuberculous inflammation was evident but occasionally the reaction to trauma passed imperceptibly into specific inflammation.

In all experiments tuberculous infection of the tissue of the chamber followed inoculation.

SUMMARY.

A technique is described whereby tissue already established in a transparent chamber in the rabbit's ear can be infected with tubercle bacilli with very little trauma.

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OBSERVATIONS ON TUBERCULOUS LESIONS IN A TRANSPARENT CHAMBER IN THE RABBIT'S EAR

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EBERT, Ahern and Bloch (1948), Ebert and Barclay (1950) and Sanders, Florey and Wells (1951) have described some of the reactions observable microscopically that follow the introduction of tubercle bacilli into the tissue of a transparent chamber in a rabbit's ear.

These observations were made with relatively low magnifications. In the present work, using the apparatus and procedure described by Sanders, Dodson and Florey (1954), an endeavour has been made to follow *in vivo* the day-to-day changes occurring in the cells that accumulate in response to the presence of tubercle bacilli, and to observe more closely than heretofore the events that take place in the neighbourhood of a growing tubercle. There are very many observations on the development of the tubercle performed by classical histological methods on fixed material. These have furnished clear pictures of the structure of a tubercle, but it has been a matter of interpretation to form an opinion about the types of cells involved, their origin, and their development. Opinions have differed widely, for example, on the origin of the epithelioid cell, though it is now generally thought that the majority of these are formed from monocytes of the blood. It is still asserted, however, that histiocytes of the tissues participate in the formation of a tubercle.

In chambers in the rabbit's ear it is easy to recognise polymorphs and macrophages and, with more difficulty, lymphocytes. With the aid of vital dyes such as vital new red or Evans' blue macrophages and histiocytes can readily be observed since they accumulate the dyes.

It has proved possible to see the earliest stage of the development of a tubercle and to produce data which may help to decide some questions of the histogenesis of tuberculous lesions.

EXPERIMENTAL.

Latent period.

A period varying from 3 to 28 days followed inoculation of tubercle bacilli into the newly formed tissue of the chamber before the first inflammatory reaction attributable to tubercle bacilli was recognised. In 14 animals in which circumstances were comparable the mean period was 10 days.

The polymorphonuclear and early macrophage reaction.

In three particularly good preparations the earliest stage of tubercle formation was seen as a collection of about 15 to 20 polymorphonuclear leucocytes in the

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interstitial tissue between capillaries. About 12 hr. later the number of polymorphs had doubled or trebled, and some leucocytes were sticking to the walls of the adjacent capillaries. The lesion continued to increase in size and cellularity and by 48 hr. a few macrophages were present.

The epithelioid tubercle.

During the second and third days of development the macrophages enlarged and became more numerous, until they were the predominant cell type. Polymorphs were found in smaller numbers, and débris was seen in the central area of the tiny tubercle, which by now was just visible when magnified 16 times. It was at this stage and by examination with the low power of the microscope that the majority of tubercles were found, probably when at least 48 hr. old. Such lesions, consisting of 100 or so " epithelioid " cells, appeared as roughly circular areas slightly more opaque than the surrounding tissue. They appeared most often adjacent to the pin but not infrequently at some distance from it. Sometimes a single lesion was seen and sometimes several developed almost simultaneously in different places.

In fixed and stained tuberculous tissue the term " epithelioid " is applied to the cells constituting such a focus and to the similar cells lying at the margins of caseous tubercles. In fixed tissues these cells can be distinguished from polymorphs, lymphocytes and fibroblasts. In the infected ear chamber the cells in positions corresponding to those seen in fixed tissue have been called epithelioid, and their identity with them is presumed. Individual epithelioid cells varied from about 25 to 50μ in diameter; their most characteristic feature was the presence of refractile granules a little larger than the granules of eosinophil leucocytes. The nucleus could not be seen but usually a clear central area, outlined by the granules, was present. With the oil immersion objective it was possible occasionally to see a waving surface membrane. Reliable criteria could not be established by which epithelioid cells could be distinguished from cells usually regarded as macrophages; cells answering to the same description were also seen around areas of blood clot in uninfected chambers. As well as this type of cell, smaller cells intermediate in appearance between monocytes and epithelioid cells were present in tubercles.

The tubercle enlarged and became more opaque as fresh macrophages collected at its margin, and in the centre granules like those in living epithelioid cells and protoplasmic fragments could be seen. Such material, which was thought to be necrotic, was present while blood still flowed through the vessels of the lesion and while the animal was tuberculin-negative. In addition to the distinct foci of cells just described, a diffuse cellular infiltration, richer in ploymorphs than the tubercle proper, soon appeared between adjacent foci.

The rôle of histiocytes.

By staining histiocytes with a vital dye before the tissue was infected it was possible to observe the behaviour of these labelled cells as a tubercle formed and spread in their vicinity. Suitable areas containing labelled histiocytes were photographed once or twice daily. As far as possible individual cells were followed at successive examinations. The marked histiocytes did not migrate towards a developing tubercle. When in the path of an advancing tubercle, they might

make slight random movements, constantly changing their shape and the pattern of the dye granules, but there was no sign of attraction towards the growing tubercle. As the tubercle approached, the number of labelled histiocytes in a given area diminished, although the total number of cells there increased. As the tubercle swept over the area of observation the labelled cells became more and more indistinct and difficult to identify (Fig. 1 and 2).

Stasis in capillaries and fusion of tubercles.

In confirmation of previous observations (Ebert *et al.*, 1948; Ebert and Barclay, 1950; Sanders *et al.*, 1951) it may be recorded that usually by the fourth to seventh day stasis which was progressive occurred in some of the vessels running through the lesion. Red cells emerged from vessels still carrying a blood flow, sometimes in sufficient numbers to form small zones of haemorrhage.

Over the next 2 or 3 days all the vessels in the area densely infiltrated with cells became occluded, and the various foci coalesced to form a single lesion often involving as much as half the tissue in the chamber.

Stasis was preceded by the adhesion of leucocytes to the endothelium of the affected vessels, and was accompanied by local oedema. This was shown by the appearance of free fluid if the coverslip and base of the chamber were bolted together loosely enough to allow of some increase in volume, and by an increase in tissue pressure and consequent reduction in the volume of circulating blood when the parts of the chamber were bolted tightly. Both stasis and oedema were considered to result from increased capillary permeability.

Caseation and the mature tubercle.

Though dead tissue appeared to form before there was any serious interference with the blood supply, necrosis increased when stasis occurred, first in the oldest parts of the lesion and later throughout the devascularised zone. At high magnifications an area of caseation appeared as a granular dirty-white zone containing fine particulate débris and closely packed structures which were shrunken and necrotic cell bodies. That this material was in fact caseous was confirmed when the animal was killed.

Thus early in the second week a fully developed tubercle was present in the

EXPLANATION OF PLATES.

FIG. 1.—Histiocytes stained with vital new red. They are mainly near the blood vessels. A tubercle is developing to the right of the picture. × 200.

FIG. 2.—The same area three days later. The necrotic area of the tubercle can be seen at the right of the picture. There do not appear to have been more than minor modifications in the distribution of the histiocytes near the developing tubercle. × 200.

FIG. 3.—Penetration of Evans' blue into an early tubercle. × 27.

FIG. 4.—A tubercle with a ring of stained macrophages. The dye has not penetrated to the centre of this lesion. × 67.

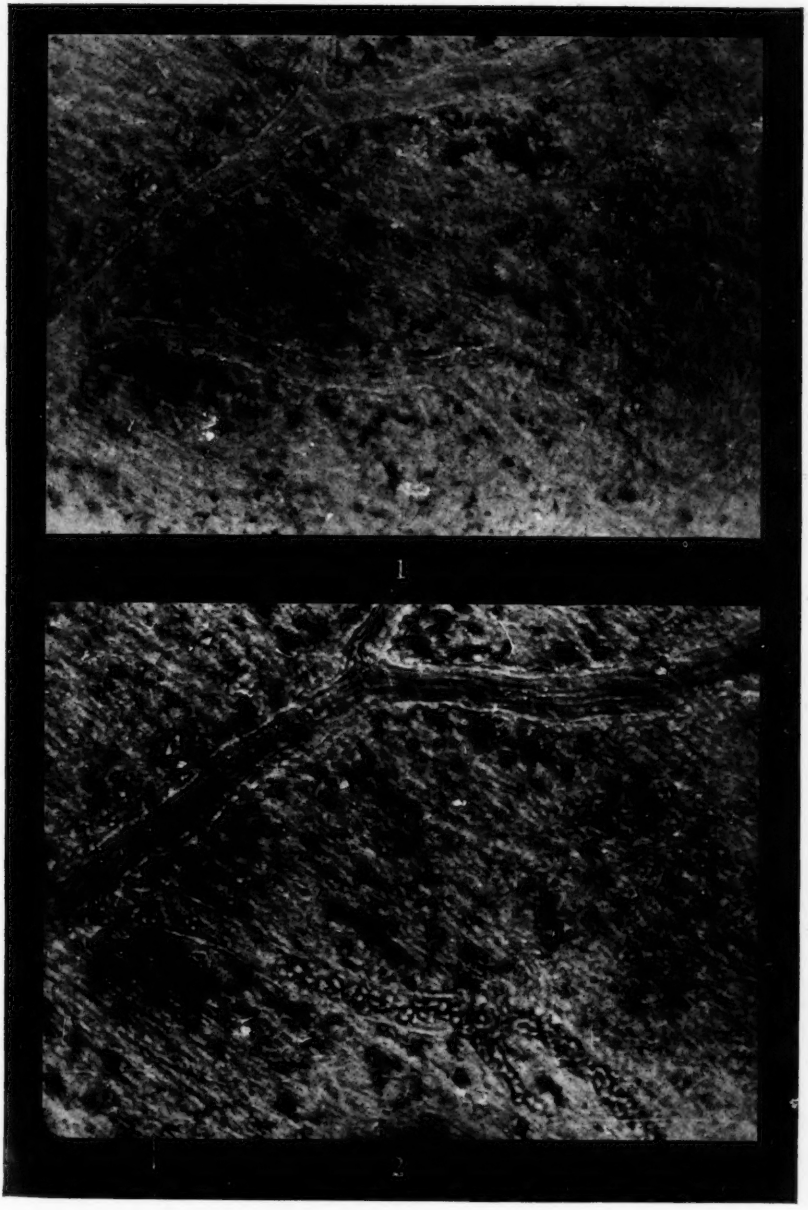
FIG. 5.—Macrophages, stained with blue dye, from the edge of a tubercle. × 167.

FIG. 6.—Tubercles in the ear chamber of a rabbit which received 2 doses of Evans' blue. The centres, which are necrotic, have been scarcely if at all stained. × 6.

Fig. 3, 4, 5 and 6 are from the same animal, taken at different stages.

FIG. 7.—Section of a lung of a tuberculous guinea-pig which had received 5 doses of 1 per cent w/v Evans' blue intravenously. The tubercles are stained blue.

FIG. 8.—Local tuberculin reaction in a rabbit ear chamber. The blood vessels are full of inspissated blood and are beginning to disintegrate at the upper part of the illustration. × 6.



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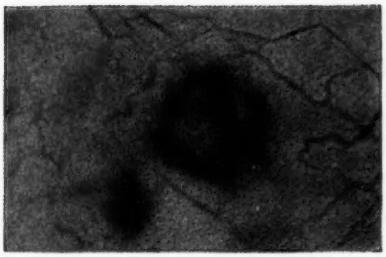

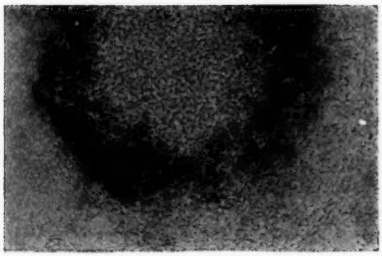

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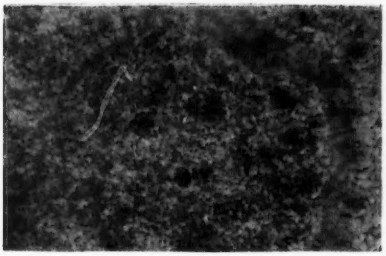

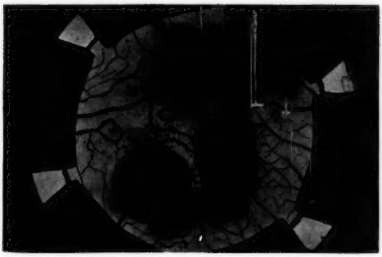

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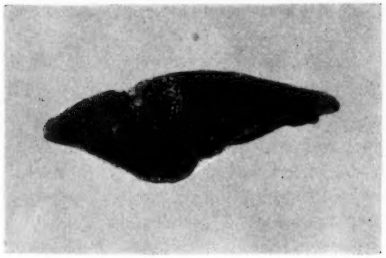

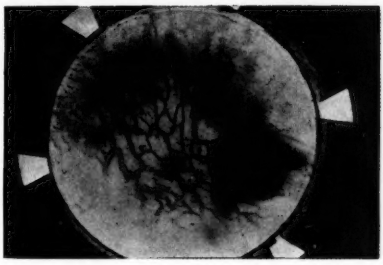

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chamber. It showed a central avascular caseous zone and at the periphery a layer some 6–10 cells deep of tightly packed epithelioid cells which gradually merged into an area less densely infiltrated by polymorphonuclear and mononuclear leucocytes. Although individual leucocytes could be seen from time to time, their total number could not be estimated because of the difficulty of identifying individuals when the cells were closely packed.

Giant cells were occasionally seen at the margins of caseous tubercles whose expansion had been arrested by chemotherapy but were not observed in rapidly extending untreated tubercles. The largest, which measured 130–140μ in diameter, appeared under low magnification as clear apparently empty spaces at the edge of the lesion. Nuclei could not be seen, and most of the cell body was occupied by coarse irregular granules like those in epithelioid cells. Surrounding the central granular area was a rim of clear cytoplasm, the movement of which could be seen when it occasionally touched the overlying coverslip. The rate and manner of this movement was identical with that shown by the peripheral membrane of rabbit monocytes in contact with glass in tissue cultures.

The formation of a giant cell was not observed. The individual giant cells which were followed either disappeared in the caseous material or became obscured by overlying epithelioid cells and could not be found again.

A tubercle did not expand at a uniform rate along its entire circumference, but grew intermittently at different sections of the edge as stasis and disintegration of blood vessels occurred. After each extension the affected area gradually became caseous and remained stationary in size while a diffuse infiltration by cells spread ahead of the caseous margin. The cellularity at the margin increased until the blood vessels in this area too went into stasis, when another increase in size rather suddenly occurred.

By the processes of stasis and thrombosis, accumulation of fresh cells at the margin of the growing tubercle and extension of the caseous zone, the whole of the tissue in the chamber was eventually converted into a caseous mass. In the untreated animal this usually occurred late in the third week after infection.

Tuberculin hypersensitivity in tissue of chamber and skin.

The relation of tuberculin hypersensitivity to the genesis of the tissue lesion has been the subject of much controversy, and it was thought that, by correlating the sequence of events studied microscopically in living tubercles with changes in the cutaneous reaction to tuberculo-protein useful information might emerge. It is, however, well known that during the course of tuberculous infections the skin of rabbits seldom becomes as sensitive to tuberculo-protein as that of some other animals, *e.g.*, man and the guinea-pig. This proved to be so in the present experiments, for cutaneous reactions to tuberculo-protein could only be elicited infrequently and irregularly from animals with a tubercle in the ear chamber.

A focal reaction around the tubercle in the chamber was thought to be a more reliable index that tuberculin hypersensitivity was present. The intravenous injection of 0·2–0·02 mg. of Purified Protein Derivative (P.P.D.) was used to elicit it. When the reaction occurred a regular sequence of events was seen. Following the injection there was a latent period of about 2 hr. during which the tissue and vessels away from the immediate vicinity of the tubercle appeared to be quite normal. Then leucocytes began to adhere in increasing numbers to the walls of the vessels about the lesion, and soon one or two vessels passed into stasis. During

the next few hours vast numbers of leucocytes adhered, and more and more vessels passed into stasis. By about the sixth hour after injecting P.P.D. many of the vessels were occluded and vascular damage reached its maximum (Fig. 8). A zone of recently emigrated leucocytes could be seen about the damaged vessels. Twenty-four hours after the injection about half the area without circulation was necrotic, and being semi-fluid was seen to pulsate with the heart beat. Foci of extravasated red cells were scattered through and around the area. In the surrounding tissues there was considerable haemorrhage and cell infiltration and many leucocytes were adhering to the vessel walls.

The relation of tuberculin hypersensitivity to tubercle formation.

Whenever the skin reacted specifically to the intradermal injection of 0·02 mg. P.P.D. a focal reaction in the chamber could be elicited by intravenous injection, but on several occasions focal reactions were elicited in the absence of cutaneous hypersensitivity. A number of animals with mature tubercles in the ear chamber failed to react either focally or in the skin to P.P.D. It seems reasonable to deduce that these animals were tuberculin-negative and that, since typical tuberculous lesions were present, tuberculin hypersensitivity is not a necessary condition for necrosis, caseation or vascular damage to occur. This differs from the view of Dienes and Mallory (1937), who claimed that hypersensitivity was necessary for the development of granulomatous lesions.

Spontaneous focal tuberculin reactions.

Familiarity with the appearances of a focal tuberculin reaction in the chamber, and the knowledge that it could occur in the absence of skin hypersensitivity, makes it possible to suggest that some of the spontaneous events that may be seen in infected chambers are manifestations of hypersensitivity. In several chambers many vessels around a tubercle suddenly went into stasis and the tissues supplied by them rapidly became necrotic and caseous. The microscopic appearances closely resembled those seen in a focal tuberculin reaction and suggested that these events were, in fact, spontaneous focal reactions. There is, however, no certainty that this interpretation is correct.

Penetration of Dyes into Tubercles.

From the point of view of chemotherapy it is important to know whether substances which may be conveyed by the blood to the region of the infection emerge from the vessels, pass into the tissues and thence into the caseous material, and remain active there. Some published observations suggest that a number of substances of differing molecular size do, in fact, enter caseous matter. Thus, iodides (Wells and Hedenberg, 1912), ferric chloride (Menkin and Menkin, 1931), simple arsenicals (Arinkin and Corper, 1916) and sulphocyanates (Corper, 1915) have been said to penetrate caseous material.

A number of observations have been made with vital dyes. Lewis (1912) found that trypan red stained the caseous material of tuberculous rabbits, and De Witt (1913) that trypan blue entered caseous material in guinea-pigs.

Using Evans' blue we have been able to confirm the entry of dye into the lesions of guinea-pigs. Evan's blue becomes fairly firmly bound to serum albumin, so it may pass out of the vessels and into the tubercle in this form. It required about

6 days for the dye to penetrate the caseous material and apparently to reach the centre of large confluent tubercles (Fig. 7). This slow penetration probably explains the results of Wells and Hedenberg (1912), who injected albumin into tuberculous guinea-pigs and could not detect it in the tubercles, as they only allowed 3 hr. for penetration.

As far as antibacterial substances are concerned, Barclay, Ebert, LeRoy, Manthei and Roth (1953) have shown that isoniazid labelled with ^{14}C in the carboxyl position reached high concentrations in solid caseous lesions in the lungs and lymph nodes of man. It is not clear whether streptomycin enters caseous material, but there is some evidence to support the idea (McDermott, 1952).

Tubercles developing in the ear chamber presented an opportunity to pursue further this question of penetration, making use of vital dyes. Evans' blue injected intravenously emerged from the blood stream in the earliest stage of the formation of a tubercle. It produced diffuse blue staining in an area which subsequently became a clearly recognisable tubercle (Fig. 3). This suggests that one of the first results of infection with tubercle bacilli is to increase the permeability to large molecules of the blood vessels in the neighbourhood, though an alternative explanation would be that the dye is adsorbed more readily in an area which is becoming tuberculous.

Though the dye is at first distributed quite evenly histiocytes, monocytes, macrophages and epithelioid cells soon take it up and become stained (Fig.5). A definite sequence of events was followed in histiocytes. About 6 hr. after injection the dye became visible as a faint diffuse colouration of the cytoplasm, which increased in intensity in the next 18 hr. During the second and third days the dye became segregated as granules, and these persisted in the cells for many weeks. Cells would stain in one part of the tissue and fail to do so in another apparently similar part. Macrophages and epithelioid cells stained more readily than histiocytes, but after a series of injections (usually 4) all the histiocytes that could be recognised in the tissue of a chamber took up dye. The epithelioid cells of small tubercles stained diffusely as a rule, granular staining being found consistently in those associated with tubercles that were healing under the influence of chemotherapy.

If two doses of dye were given at an interval of 3–4 days when a tubercle was rapidly expanding, two zones of intense staining appeared at its margin, the zone from the second injection lying outside that from the first. This was well shown by using dyes of different colours for two injections.

If dye was administered when a mature tubercle with a caseous centre was present it was not possible to detect it microscopically in the caseous material, although some dye was probably present, for if a large lesion was examined with the naked eye, or if the coverslip was removed and a loopful of the caseous material scraped up, a faint colouration could be seen. It was clear that the dye was much more concentrated in the epithelioid cells than in the caseous material (Fig. 4 and 6).

In addition to the phagocytic cells, the connective tissue in an early tubercle, or at the margin of a mature lesion, stained with the dye. The staining was diffuse and was not apparently related to the direction of the collagen fibres. The stained material seemed probably to be the ground substance of the fibrous tissue, taking up and losing the dye more rapidly than the cells. When, as sometimes occurred, a tubercle was expanding at one segment of its edge and not elsewhere, staining

of the connective tissue was restricted to that segment. It was not possible to decide whether this was the result of increased capillary permeability in that particular area or of an increased affinity of the ground substance for the dye.

It seems clear that substances with large molecules can emerge from the blood stream in the neighbourhood of an acute tubercle. Macroscopic observations on tubercles in the lungs of guinea-pigs give the impression that Evans' blue has diffused fairly evenly into the lesions. Microscopic observations on small tubercles developing in chambers in the rabbit's ear show, however, that they can enter the margin of a tubercle and be taken up by the phagocytic cells present there, but that they do not (at least with this dye) penetrate readily into the caseous material itself. These observations would give some support to the view previously put forward that an anti-mycobacterial substance with a large molecule might have advantages in the treatment of tuberculous disease (Florey, 1952). If it behaved in the same way as the dyes just described the phagocytes would come to contain within their cytoplasm a highly concentrated antibacterial substance, and thus would be in a better position to kill phagocytosed bacilli. Possibly one of the best solutions to the chemotherapy of tuberculosis will be provided by a combination of a substance which diffuses freely into dead material with another which is taken up and concentrated by the phagocytic cells.

SUMMARY.

A description is given of the development of tuberculosis in a transparent chamber in the rabbit's ear.

Histiocytes of the connective tissue apparently play no part in the formation of a tubercle.

Vital dyes enter tubercles at an early stage. They are taken up by macrophages at the edge of the lesions.

A focal tuberculin reaction is described.

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THE NECROTIC LESION IN GUINEA-PIG SKIN PRODUCED BY CERTAIN HUMAN SERA.

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IT was found that when 0·1 ml. of fresh unclotted blood from a patient with rheumatoid arthritis was injected intradermally in a guinea-pig necrosis followed, while blood from a healthy subject caused only redness and swelling (Goodman and Lovell, unpublished observation). The results were the same when serum or heparinized plasma was used. Sera from other healthy subjects and from patients with a variety of diseases were then examined. While macroscopically detectable necrosis occurred with most fresh sera from patients with active polyarthritis, it also occurred with some fresh sera from patients with unrelated diseases and occasionally with fresh sera from apparently healthy subjects. Sera which produced necrosis when injected into the skin of the guinea-pig did not produce necrosis when injected intradermally into the cat, rat, mouse, rabbit, sheep or man. In this paper we describe the lesions produced by human sera in guinea-pig skin. The distribution and properties of necrotizing activity in human sera are described in the following paper.

MATERIALS AND METHODS.

Human blood was obtained by venipuncture and guinea-pig blood by heart puncture of normal animals. After clotting, the blood was centrifuged. The serum was injected within 6 hr.

Albino female guinea-pigs weighing 250–600 g., fed on Bruce and Parkes (1947) pellet diet and cabbage, were used for the tests. After depilation with barium sulphide paste, intradermal injections of 0·1 ml. were made in the back or belly, using a tuberculin syringe and gauge 26 needle. Lesions for histological study were excised and stained with haematoxylin and eosin, and sometimes with other stains.

Reactions to about 80 sera were observed, and lesions from 25 guinea-pigs were examined histologically. To study the development of reactions, injections of the same serum were made in the same animal at intervals before it was killed. To compare reactions to different sera, replicate injections were made in the same animal. Injections were also made in the skin of dead guinea-pigs.

The local effect of serum on the skin circulation was studied in 12 guinea-pigs. Serum was injected intradermally. Between 5 min. and 24 hr. later, 5–15 ml. India ink was injected into the heart and the animals were then killed. The distribution of carbon in relation to injection sites was noted. Some skins were then excised, cleared in methyl salicylate, and the lesions studied with a dissecting microscope.

For convenience, the following terms are used:

Necrotizing sera: sera producing naked-eye necrosis after 24 hr.

Non-necrotizing sera: sera not producing naked-eye necrosis after 24 hr.

Inactivated sera: sera heated to 56° for 45 min.

*In receipt of a grant from the Endowment Fund of St. Mary's Hospital; Scholar of the Royal Australasian College of Physicians.

RESULTS.

Naked-eye Appearances.

Necrotizing sera.—After 10 min. the intensely pale wheal produced by the injection develops a slaty-blue colour. The lesion rapidly becomes purplish and in 15 min. is 6–8 mm. diameter. After 24 hr. the lesion has a well-defined mauve or grey central area 3–9 mm. diameter, which may be mottled, surrounded by a slightly swollen zone of erythema up to 20 mm. diameter (Fig. 1*a*). The epithelium over the central area is friable and, if it is ruptured, serous fluid exudes. The hair in this area does not grow. Subsequently a scab forms and persists for 2–3 weeks. The site of the lesion is finally marked by a scar.

Non-necrotizing and inactivated sera.—There may be no visible abnormality, or more commonly a swollen red area which at 24 hr. may be 20 mm. diameter (Fig. 1*b*).

Microscopic Appearances.

Necrotizing sera.—Within 5 min., over a clearly demarcated area, the epidermal cells are swollen and pale. At first the nuclei are unaltered, but by 5 hr. they are pyknotic or karyolytic. At 24 hr. there is a thin layer of necrotic epidermis usually separated from the dermis by exudate. The hair follicles are less affected, though by 24 hr. they also are necrotic.

In the dermis, the collagen, elastic and reticulin fibres remain unchanged, but at 24 hr. the fibrocytes are pale, swollen and necrotic. Necrosis of muscle fibres in the panniculus carnosus is a striking feature as early as 5 min. after injection. The damage within fibres is segmental. Parts of affected fibres are swollen and stain more deeply with eosin, and other parts show disruption of the sarcoplasm with collapse of the sarcolemmal sheath (Fig. 6). The sarcolemmal nuclei, however, of both swollen and collapsed segments are unaltered. Throughout the lesion the affected muscle fibres are separated, presumably by oedema. The endomysial fibrocytes, unlike those in the dermis, appear unchanged.

Vascular changes are also conspicuous within 5–10 min. Most of the capillaries and veins are dilated and engorged and some show mural thrombosis (Fig. 3 and 5).

DESCRIPTION OF PLATES

FIG. 1.—Lesions in the skin of a guinea-pig injected 24 hr. previously with (*a*) necrotizing serum (*b*) non-necrotizing serum. (× 1.)

FIG. 2.—Skin of a guinea-pig cleared with methyl salicylate. Necrotizing serum was injected on the left, and non-necrotizing serum on the right 15 min. before perfusion by intracardiac India ink. On the left there is a gap in the normal pattern of ink-filled vessels. (× 2.)

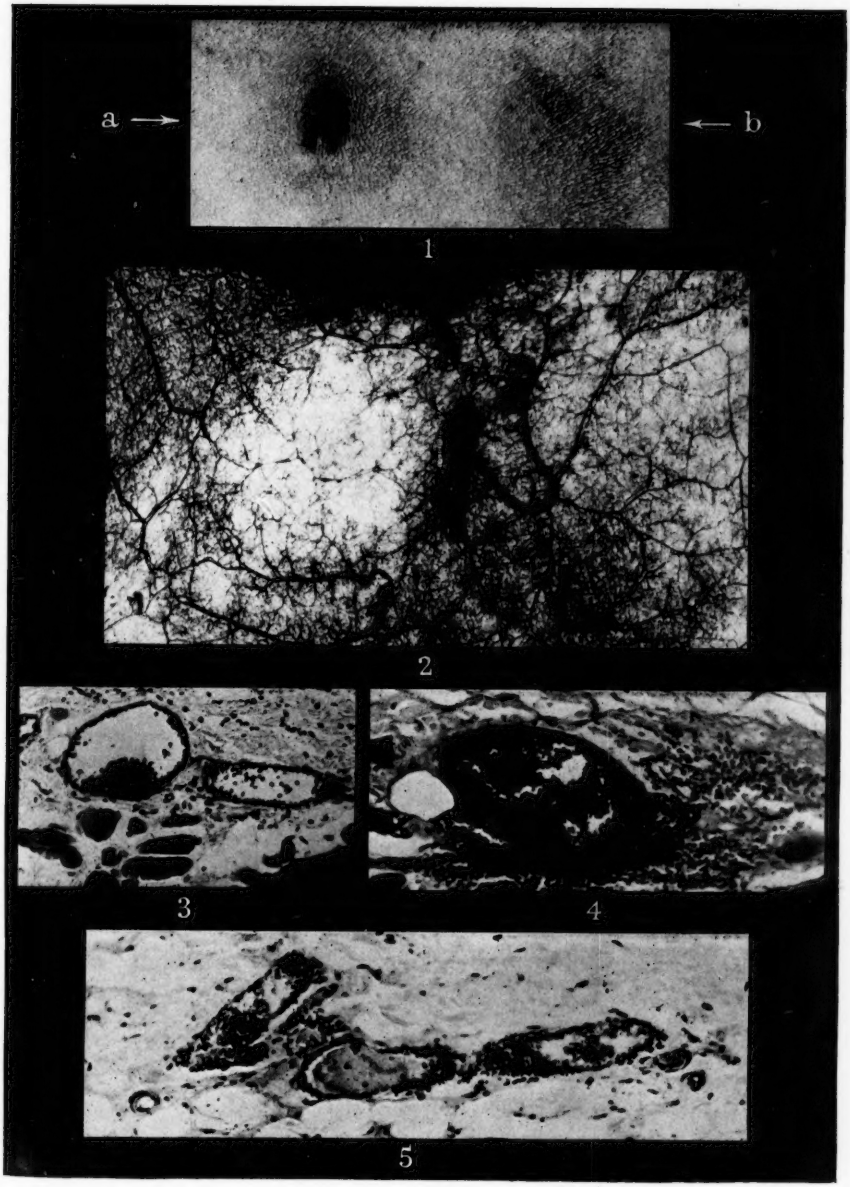
FIG. 3.—Section through the skin of a guinea-pig injected with necrotizing serum 24 hr. before death, showing a vein with a mural thrombus. There are also foci of necrosis in the muscle fibres of the panniculus carnosus. (× 125.)

FIG. 4.—Section through the skin of a guinea-pig injected with necrotizing serum 24 hr. before death, showing a dilated vein with a mural thrombus, necrosis of the vessel wall and surrounding haemorrhage. (× 125.)

FIG. 5.—Section through the skin of a guinea-pig injected with necrotizing serum 12 min. before death, showing dilated thrombosed veins. (× 125.)

FIG. 6.—Sections through the panniculus carnosus of a guinea-pig. (*a*) At a site injected 12 min. previously with a necrotizing serum. (*b*) At a site injected at the same time with non-necrotizing serum. (× 125.)

FIG. 7.—Panniculus carnosus of a guinea-pig injected 5 min. after death with (*a*) necrotizing serum, showing muscle lesions and (*b*) with non-necrotizing serum. (× 125.)



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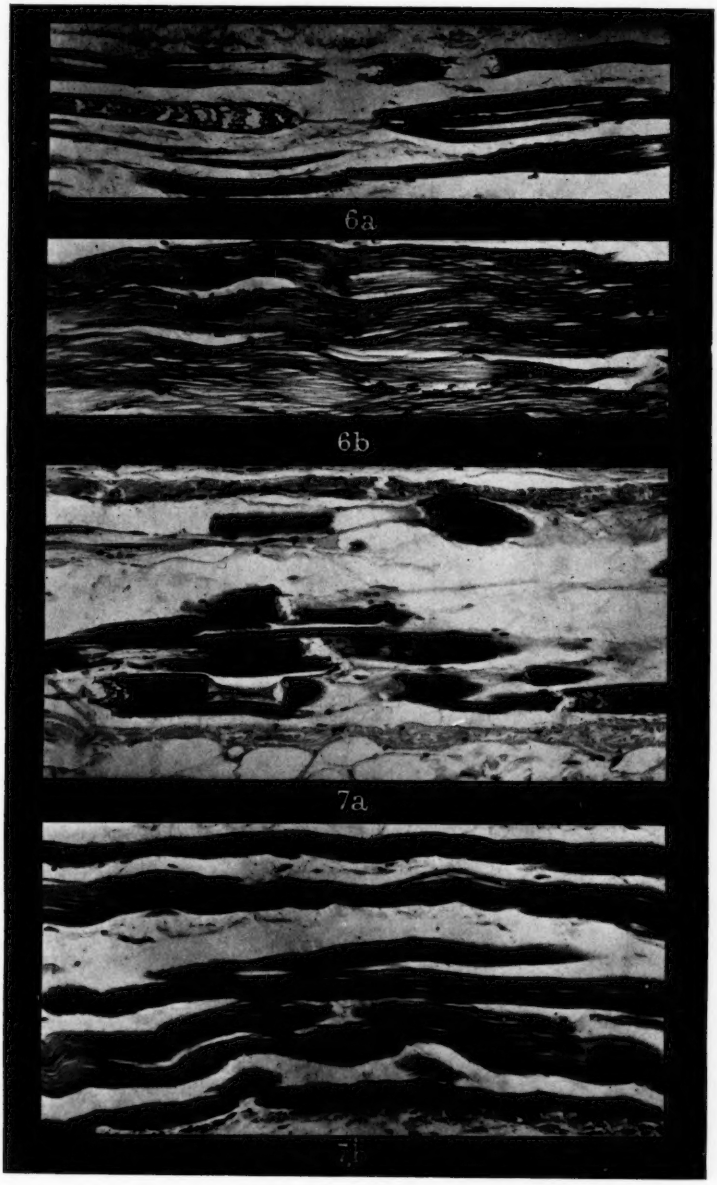

Lovell, Pryce and Boake.

In places this causes complete occlusion and the vessels thus affected may be necrosed. Minute focal haemorrhage occurs (Fig. 4). Polymorphs can be seen in the thrombosed vessels in the early stages, but later these migrate into the surrounding tissue.

The lymphatics in the early stages are filled with oxyphil fluid and some of the lining cells have pyknotic nuclei. By 24 hr. the lymphatics have been largely destroyed. The arteries at 24 hr. appear unchanged.

In the dermis surrounding the lesion there is an inflammatory reaction.

By 48 hr. there is a central area in which all cellular components of the skin are necrosed, though in the dermis collagen fibres still appear intact. In the panniculus adiposus there are foci of fat necrosis. In the panniculus carnosus the damaged muscle fibres are disintegrating, and some are invaded by polymorphs and macrophages; peripherally there is sarcolemmal proliferation and leucocytic infiltration. In lesions 6 days old the necrotic dermis is shrunken and invaded by granulation tissue and the adjacent epithelium is thickened. By the nineteenth day there is scar tissue in the dermis, fat necrosis is conspicuous in the panniculus adiposus, and in the panniculus carnosus there are numerous small muscle fibres.

Non-necrotizing sera.—Although on naked-eye examination these lesions do not appear necrotic, on microscopical examination there is necrosis of epidermal cells. Within an hour there is moderate vascular dilatation with diapedesis of red cells and polymorphs and swelling of fibrocytes. The polymorphs increase in number and by 24 hr. they are numerous throughout the lesion. Thrombosis has not been observed. Changes resembling those produced by necrotizing sera were seen in some muscle fibres; they were never numerous and some may have been artifacts, for their incidence was reduced if extreme precautions were taken to handle the tissues gently. In reactions whose macroscopic classification at 24 hr. was in doubt, both muscular and vascular lesions were sometimes found.

Inactivated sera.—These produced mild inflammatory reactions.

Guinea-pig sera.—The inflammation was even less than that evoked by inactivated human sera.

India Ink Preparations.

Intracardiac injection of India ink caused greying of the skin, which included sites previously injected with non-necrotizing and inactivated sera, but spared sites injected with necrotizing sera. In cleared skins, the vessels at sites injected with non-necrotizing or inactivated sera were filled with ink. The appearance of sites injected with necrotizing serum depended on the time elapsing between injection of serum and injection of ink. When the interval was 5–10 min., they appeared as clear areas amid the surrounding meshwork of ink-filled vessels (Fig. 2). Injection sites 15 min. to 24 hr. old appeared as areas of grossly dilated vessels filled with coagulated blood. At the margins of these lesions, the blood-filled vessels were continuous with the smaller normal skin vessels containing ink. In these older lesions a little carbon was seen in some arteries but not in the minute vessels and veins.

Changes Produced in the Dead Guinea-pig.

A depilated guinea-pig was killed with ether and kept in an incubator at 37°. Intradermal injections of 0·1 ml. of 3 different necrotizing sera and 1 non-necrotiz-

ing serum were made 15 min. after death. The same volumes of the same sera, heated at 56° for 45 min., were injected in corresponding sites in the opposite side of the animal. One hour later the skin was excised and fixed in formalin. Microscopic examination showed segmental muscle lesions, indistinguishable from those already described in the living animal, in the 3 sites injected with necrotizing sera, but not in the other sites. Other experiments showed that, as in the living animal, these muscle lesions occur within 5 min. of injection of serum (Fig. 7).

In an experiment to measure the period after death during which muscle lesions could be produced, a necrotizing serum was injected at intervals. When the interval between death and injection was 2–30 min., muscle lesions were plentiful; they were slight or absent when the interval was 1 hr.

DISCUSSION.

Of the changes which follow injection of necrotizing sera, those in the blood vessels are particularly striking. The transient initial pallor is due to emptying of the minute skin vessels and to cessation of blood flow through them. This is not attributable to compression of the vessels by the fluid injected for ink injected into the heart flowed freely through the control lesions. It is probably due to vaso-constriction. The next phase consists of gross dilatation, engorgement and thrombosis. The early appearance of mural thrombi presumably indicates endothelial damage.

The early and complete obstruction of blood flow led us to consider whether the other changes in the skin were solely due to ischaemia. The very early occurrence of muscle lesions in the living animal made this unlikely, and their occurrence in the dead animal provided evidence of a direct effect of serum on muscle substance. It seems, therefore, that at least two tissues are directly injured by necrotizing serum; the capillaries and veins, and striated muscle fibres.

We have designated reactions as necrotic or non-necrotic according to the naked-eye appearances and have classified sera accordingly. However, microscopic studies suggest that this distinction is not absolute, inflammation and muscle lesions appearing in at least some reactions which are not macroscopically necrotic. There appears to be a graded response from slight inflammation to gross necrosis.

There is nothing specific in the vascular or muscle changes in the lesions which might indicate the nature of the tissue injury. Mural thrombi are early features of other experimentally induced skin reactions, such as that caused by the injection of serum containing Forssman antibody in guinea-pigs (Redfern, 1928), and the Shwartzman reaction in rabbits (Shwartzman, 1937). The muscle change resembles the coagulative necrosis which follows various types of injury.

SUMMARY.

Some fresh human sera cause necrosis when injected in the guinea-pig's skin, others cause only redness and swelling. Necrotizing activity is lost in sera heated at 56° for 30 min.

The necrotic reaction has two conspicuous features which appear within 10 min. of injection, engorgement and dilatation of the capillaries and veins, with mural thrombi, and segmental necrosis of muscle fibres in the panniculus carnosus. The muscle changes are also produced by injection of necrotizing serum into the skin of a dead guinea-pig maintained at 37°.

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THE NECROTIZING ACTIVITY OF HUMAN SERA FOR GUINEA-PIG SKIN.

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THE toxicity of some human sera for the guinea-pig has long been recognized. Subcutaneous injection of fresh serum causes local inflammation and sometimes necrosis (Uhlenhuth, 1897 ; Delaunay, Robineaux and Lebrun, 1950) ; intravenous injection causes an anaphylactoid reaction (Ramsdell and Davidsohn, 1929). We have described in the preceding paper the macroscopic and histological features of the lesion which follows intradermal injection of fresh serum or plasma.

Little appears to be known of the toxic factor in fresh serum except that it is thermolabile (Doerr and Moldovan, 1907 ; Delaunay *et al.*, 1950). When fresh serum was heated, Doerr and Moldovan found a rough correlation between loss of complement and loss of toxicity on intravenous injection. The intravenous toxicity of stored sera has been more extensively studied. It was attributed by Taniguchi (1922) to heterophile (Forssman) antibody but Davidsohn and Ramsdell (1930) showed that it was not entirely accounted for by this.

Our observations have been made with fresh human sera, and concern the distribution and properties of the active component of certain sera which cause necrosis when injected intradermally in the guinea-pig.

MATERIALS AND METHODS.

Albino female guinea-pigs were used. They weighed 250–600 g., and were fed on Bruce and Parkes' (1947) pellet diet and cabbage. They were depilated with barium sulphide paste.

Serum was obtained from blood clotted in glass tubes. Unless otherwise stated, 0·1 ml. was injected within 6 hr. with a tuberculin syringe and a gauge 26 needle into the skin of the back.

A lesion was designated as necrotic if on the day following injection of serum there was a central area, with bluish-red or yellow discolouration, in which the hair stumps had not grown. The diameter of necrosis was recorded as the mean of the maximum diameter and that at right angles to it.

Estimation of complement.

Serum was diluted in saline 1/5, 1/10, 1/15 . . . 1/50. To the diluted serum an equal volume of a 3 per cent suspension of sheep's red cells sensitized with 5 M.H.D. of horse anti-sheep serum (Burroughs Wellcome) was added. The tubes were read after incubation for 30 min. at 37°. The last tube in which complete haemolysis had occurred was taken as the end-point.

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Estimation of agglutinins.

Anti-sheep and anti-guinea-pig agglutinins were estimated by preparing 2-fold serial dilutions of inactivated sera in saline. To each tube was then added an equal volume of a 3 per cent suspension of the appropriate red cells. The tubes were placed in the refrigerator overnight before being read. The last tube showing complete agglutination was taken as the end-point.

Estimation of haemolysin.

Equal volumes of a 3 per cent suspension of washed sheep's red cells were added to serial 2-fold dilutions of inactivated serum in saline, and incubated for 30 min. at 37°. One volume (0·25 ml.) of saline containing 5 M.H.D. of complement (guinea-pig serum) was then added and the tubes were examined after incubation for 30 min. at 37°. The last tube in which complete haemolysis had occurred was taken as the end-point.

RESULTS.

Distribution of necrotizing sera.

Sera from 74 subjects were examined. The results are summarised in Table I. Of 16 sera from apparently healthy subjects, 2 caused necrosis. Of the 20 sera from patients with active polyarthritis, all but 1 caused necrosis, while sera from 2 patients whose arthritis was inactive did not. Necrosis was also caused by 21 out of 36 sera from other ill patients.

TABLE I.—*Distribution of Necrotizing Activity in 74 Sera.*

Diagnosis.	Number of subjects.	Result.	
		Necrosis.	No necrosis.
Healthy	16	2	14
Active polyarthritis	20	19	1
Inactive polyarthritis	2	0	2
Peptic ulcer	7	2	5
Polyarteritis nodosa	4	4	0
Essential hypertension	2	2	0
Malignant hypertension	2	2	0
Myocardial infarction	4	4	0
Bronchial carcinoma	2	1	1
Chronic rheumatic carditis	2	1	1
Hyperthyroidism	2	1	1
Intermittent claudication	2	0	2
Thrombophlebitis	1	1	0
Nephritis (Ellis Type II)	1	0	1
Congenital renal cyst	1	0	1
Post-mastectomy	1	1	0
Cerebral tumour	1	0	1
Systemic lupus erythematosus (in remission)	1	0	1
Penicillin reaction	1	1	0
Dermatitis and asthma	1	0	1
Chronic bronchitis	1	1	0

The variation in response to the same serum in different guinea-pigs.

The variation in the mean diameter of the lesion produced by the same dose of the same serum in different guinea-pigs, and the variation between different sites on the same animal's back were studied. In a standard type of multifactorial experiment, using 5 guinea-pigs and 12 sites on each, the difference in diameters between guinea-pigs was significant ($0{\cdot}01 < p < 0{\cdot}05$). There was also a significant difference in the diameters of the lesions at different sites but this was due to the small lesions which occurred above the level of the angles of the scapulae.

When lesions were produced in an area bounded above by the angles of the scapulae, below by the iliac crests, and laterally by a line 6 cm. from the mid-line, there was no significant site-to-site variation. In subsequent studies this area alone was used for injection. We later found that about 10 per cent of guinea-pigs were relatively or absolutely refractory to known necrotizing sera. Animals weighing over 400 g. gave the most consistent responses.

The relation between the volume of serum injected and the mean diameter of the lesion.

Quadruplicate injections were made, in 10 guinea-pigs, of the following volumes of the same serum : 0·02, 0·05, 0·10 and 0·15 ml. The mean diameters of the lesions were recorded on the following day. The relation between the logarithm of the dose (ml.) and the mean diameter of necrosis is shown in Fig. 1. The

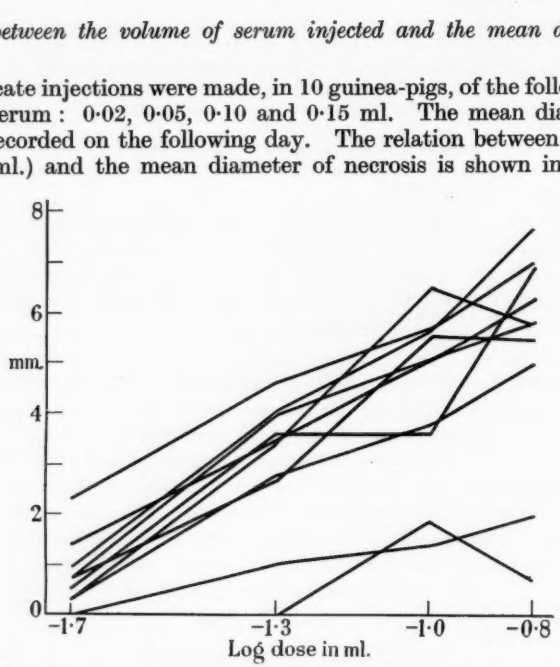

FIG. 1.—Mean diameters of necrosis related to logarithm volume of serum injected in 10 guinea-pigs.

diameter of necrosis was roughly proportional to the logarithm of the volume injected, except in 2 animals which were relatively refractory. Two further observations provide evidence that the necrotic response is related to the dose of serum injected. Firstly, some non-necrotizing sera may not produce naked-eye necrosis when 0·1 ml. is injected, but do so when 0·2 ml. is injected. Secondly, a serum which caused no necrosis after injection of 0·1 ml. produced necrosis when freeze-dried and reconstituted in water to half its original volume.

The variation in necrotizing activity of individual sera.

Sera from 14 subjects were injected repeatedly into different guinea-pigs, some of them on as many as 30 occasions during periods of up to 18 months. The 8 initially necrotizing sera have never failed to cause necrosis, except in refractory animals. Of the 6 initially non-necrotizing sera, 2, each on one occasion, caused

necrosis. On one such occasion the subject was suffering from a respiratory infection.

The appearance of necrotizing activity in non-necrotizing sera.

A volunteer, whose serum was non-necrotizing, was injected intramuscularly with 1·0 ml. of T.A.B. vaccine (Parke Davis). He developed an acute local inflammatory reaction and fever. His serum was examined at intervals by making triplicate injections of 0·10, 0·15, and 0·20 ml. in randomized sites on the backs of 2 guinea-pigs. The reactivity of the guinea-pigs was established by injecting a known necrotizing serum in the same way at the same time in the same pig (Fig. 2). The volunteer's serum became necrotizing within 24 hr., but after 14 days it had lost its necrotizing activity.

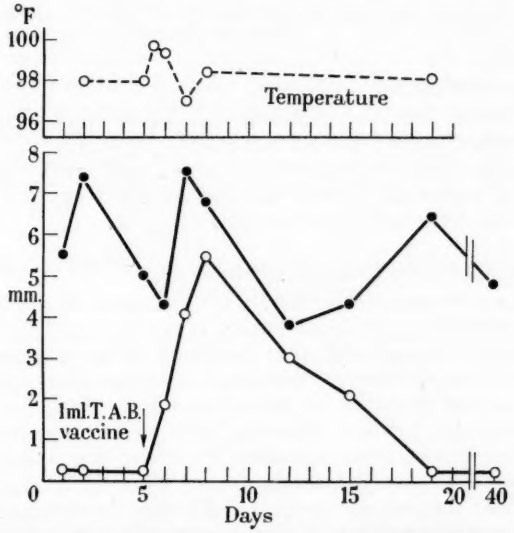

FIG. 2.—Necrotizing activity induced in serum by injection of typhoid vaccine. Open circles show mean diameters of necrosis produced by serum of vaccinated subject and above his temperature. Black dots show diameters of necrosis produced by serum of patient with polyarteritis nodosa.

The effect of cortisone on necrotizing activity.

Sera from 4 patients with polyarteritis nodosa and from one with rheumatoid arthritis were tested while they were receiving cortisone 25 to 200 mg./day. All caused necrosis. Necrosis was not prevented by treating a guinea-pig with 25 mg. of cortisone daily for 5 days before the injection of necrotizing serum.

Physical Properties of Necrotizing Sera.

Effects of temperature and storage.—Sterile necrotizing serum is inactivated by storage for 24 hr. at room temperature, and after 30 min. heating at 56°. It survives 3–4 days at 0°, and for a week at least at −15°.

Dialysis.—Necrotizing activity in serum is not dialysable against running tap-water for 12 hr. at 4°.

Dilution in saline.—Six necrotizing sera were diluted with 0·9 per cent saline 4/5, 3/5, 2/5, 1/5. Necrotizing activity was always lost at a dilution of 1/5 and generally at 2/5.

Biological Properties of Necrotizing Sera.

Relation to human anti-guinea-pig agglutinin.

Human sera frequently agglutinate guinea-pig red cells to a low titre. The relation of this agglutinin to the necrotizing activity of serum was studied. Firstly, it was found that heating serum to 56° for 30 min. did not result in the loss of guinea-pig agglutinin, whereas it caused loss of necrotizing activity. Secondly, when guinea-pig agglutinin was removed from necrotizing serum by absorption with packed guinea-pig erythrocytes at 0°, necrotizing activity persisted.

Effect of adsorption with guinea-pig tissues.

The findings in the previous experiment made it appear unlikely that the necrotizing agent was a species-specific anti-guinea-pig antibody. This possibility was further examined by mixing necrotizing serum with equal volumes of suspensions of ground-up guinea-pig muscle and kidney for 1 hr. at 0°. Necrotizing activity was not lost, although agglutinin was removed.

Relation of necrotizing activity to Forssman antibody.

Serum containing Forssman antibody produces necrosis when injected intradermally into the guinea-pig (Redfern, 1926 ; 1928), and the lesion thus produced has some features in common with that produced by human necrotizing serum (Lovell, Pryce and Boake, 1954). Furthermore, trypan blue appears to modify some tissue mechanism common to these reactions. Jungeblut and Newnan (1930) showed that the necrosis following intradermal injection of Forssman antiserum, like that following an injection provoking the Arthus phenomenon, was inhibited if the injection was made in a site previously infiltrated with trypan blue. We found that the reaction to 0·1 ml. of human necrotizing serum was also inhibited when it was injected into a site injected 15 min. previously with 0·5 ml. of 1 per cent trypan blue. The following differences, however, establish that necrotizing activity is distinct from Forssman antibody.

(i) The necrotizing activity of Forssman antiserum is not lost, or even reduced, by heating at 56° for 30 min., or on prolonged storage at 0°.

(ii) The necrotizing activity of Forssman antiserum is removed by absorption with sheep erythrocytes (Redfern, 1926). There was no loss of necrotizing activity of human serum after absorption of heterophile agglutinin with sheep erythrocytes.

(iii) In human sera, there is no relation between the necrotizing activity and the content of anti-sheep agglutinin and haemolysin (Table II).

The relation of necrotizing activity to complement.

Both the necrotizing activity of human serum and the complement content are lost on heating to 56° for 30 min. The relation of complement to necrotizing activity was investigated in the following experiments :

A precipitate was prepared by adding crystalline hen ovalbumin (Armour Laboratories) to 1 ml. of anti-hen ovalbumin rabbit serum until no further precipitate formed. The packed precipitate was washed with saline and then mixed and incubated with 2 ml. of necrotizing serum for 30 min. at 37°. This treatment resulted in the loss of both complement and necrotizing activity. The addition of crystalline ovalbumin alone (1 mg./ml.) did not result in loss of activity. When the necrotizing serum was decomplemented with other antigen-antibody complexes, *i.e.*, precipitates produced by interaction of human serum and anti-human rabbit serum, and of rabbit and anti-rabbit fowl serum, necrotizing activity was also lost.

The hen albumin anti-hen albumin precipitate, after being used for decomplementing a necrotizing serum as described above, was suspended in 1·0 ml. saline. This suspension was then injected into a guinea-pig. Necrosis did not occur.

Necrotizing sera were decomplemented by the addition of antigen-antibody precipitate or by heating. Attempts were then made to restore necrotizing activity by the addition of human or guinea-pig sera rich in complement. We never succeeded in restoring necrotizing activity by restoration of complement. This failure was not attributable to dilution of the serum, for necrosis still occurred when necrotizing serum containing complement was diluted with decomplemented non-necrotizing serum so that the final complement titre was the same as in the test system.

TABLE II.—*Sheep Red Cells Agglutinin and Lysin Titres of Necrotizing and Non-necrotizing Sera.*

Agglutinin.		Lysin.	
Necrotizing serum.	Non-necrotizing serum.	Necrotizing serum.	Non-necrotizing serum.
< 2	2	32	16
< 2	4	32	32
< 2	4	64	32
8	8	48	48
8	8	48	48
8	16	64	64
8	32	64	64
16	32	96	128

To inactivate C3, necrotizing serum was treated with " zymosan," prepared from yeast according to the method of Pillemer and Ecker (1941). Another serum was treated with ammonia to inactivate C4 (Gordon, Whitehead and Wormall, 1926). In each case the necrotizing activity was lost.

The complement titres of a group of necrotizing and non-necrotizing sera were determined. There was no significant difference in complement titres between the 2 groups (Table III).

TABLE III.—*Complement Titres of Necrotizing and Non-necrotizing Sera.*

Mean titres.		Difference between means ± standard error.	P.
Necrotizing sera.	Non-necrotizing sera.		
40	35	5±3·32	0·1

Based on 10 sera of each kind.

DISCUSSION.

Necrotizing activity in fresh human serum appears in a variety of diseases whose common component appears often, but not always, to be the presence of inflammation.

The factor in the serum which produces necrosis has not been characterized chemically. Its thermolability and its inability to pass through cellophane suggest that it may be associated with a protein. It is probably a component of normal serum which is increased in many diseases. It does not appear to be a species-specific anti-guinea-pig antibody and it is distinct from Forssman antibody.

The relation of necrotizing activity to complement is uncertain. Measures removing all or part of complement, namely, storage, heating, treatment with antigen-antibody precipitates, ammonia and zymosan, remove all necrotizing activity. On the other hand, all non-necrotizing sera tested contained complement and some were rich in it; necrotizing activity did not appear to be related to complement titres and it could not be restored to de-complemented serum by restoration of complement. It seems therefore that necrotizing activity is not identical with complement as measured by the haemolytic method. It might be related to a specific component of complement and this possibility merits further investigation.

The necrotizing factor appears to be another example of an ill-defined group of substances with diverse properties, which appear, or increase in concentration in serum in the active stages of a variety of diseases. Of these substances, C-reactive protein (Tillett and Francis, 1930) has been best studied. Others include Hughes' (1937) " anti-immunity " factor, Tillett's (1937) streptococcus bactericidal factor, non-specific hyaluronidase inhibitor (Friou and Wenner, 1947), serum mucoprotein (Kelley, Good and Glick, 1950) and complement. The biological significance and possible inter-relationships of these substances remain to be discovered.

SUMMARY.

Fresh sera from patients with rheumatoid arthritis and also sera from patients with a variety of other diseases in which inflammation is often present, produce necrosis when injected intradermally in the guinea-pig.

The necrotizing factor is not dialysable. Necrotizing activity is lost when serum is de-complemented but activity is not restored by restoration of complement. The factor does not appear to be an anti-guinea-pig antibody nor is it related to Forssman antibody.

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THE GROWTH OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS IN THE DEVELOPING CHICK EMBRYO.

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SOON after the virus of L.C.M. was isolated by Armstrong and Lillie (1934) it was shown by Bengtson and Wooley (1936) that it could be cultivated and passed in the chorio-allantois of the developing chick embryo. This finding has been confirmed by Tubaki (1940), Milzer and Levinson 1942) and Kraft and Gordon (1947).

None of these workers, however, studied the pattern of growth of the virus in the chorio-allantois or investigated whether the chick embryo could be infected by other routes of inoculation. Kraft and Gordon (1947) noted briefly that virus multiplied in the yolk-sac but did not indicate that spread elsewhere took place.

MATERIALS AND METHODS.

Virus strains.

W.E. strain.—This strain has been passed countless times through mice and guinea-pigs since it was first isolated by Rivers and Scott (1936). It was kindly supplied by Dr. F. O. MacCallum.

84B strain.—This strain was isolated from a guinea-pig inoculated with the cerebrospinal fluid of a patient suffering from aseptic meningitis but its origin was probably the inoculated animal as no serological response could be shown to L.C.M. virus in the patient's serum.

These strains were chosen amongst the many available from human cases and from infected house mice because the W.E. strain was always lethal to guinea-pigs while the 84B strain produced only a very mild fever and never death in these animals. In this laboratory the 84B strain has been passed only in mice while the W.E. strain has been passed in both mice and guinea-pigs.

Stock suspensions of both viruses were made by grinding up brains from infected mice in ten times their volume of Hartley broth. After centrifugation at 3000 r.p.m. for 10 min. the supernatant was removed, distributed in small amounts and kept frozen at $-35°$ until required. At this temperature the virus kept its titre for 3–4 months and after a year had only lost about 50 per cent of its infectivity.

As the usual $I.D._{50}$ titres of virus obtained in this way varied between 10^{-4} and 10^{-5} a suspension of the W.E. strain was prepared from infected guinea-pig spleen in a similar manner when a virus of higher titre was needed.

When required for use the stock virus suspensions were diluted in Hartley broth to give the required infective dose. One thousand units each of penicillin and streptomycin were added to the inoculum for injection by routes other than the chorio-allantoic one.

Titration of virus.

Mice were observed for 12 days after inoculation ; death after the 6th day, the production of a typical fit or definite ruffling of the fur of an animal were taken as signs of virus infection. Death could not be taken as the end-point as mice, although having fits on being

spun by the tail, or appearing ill, sometimes recovered. As no macroscopic lesions were produced in fertile eggs their virus content had to be determined by mouse inoculation.

The $I.D._{50}$ dose of virus was calculated by the method of Reed and Muench (1938), five 8–12-week-old mice being inoculated intracerebrally with 0·03 ml. of each 10-fold dilution.

Complement fixation technique.

Complement fixation tests were performed by the routine technique used in this laboratory (Duncan, 1951) for the demonstration of L.C.M. antibody ; in the search for soluble antigen in tissue extracts, preparations were titrated against a positive antiserum as had been suggested by Smadel and Wall (1941) for the identification of the virus.

Inoculation and harvesting of the developing chick embryo.

Eggs incubated for 8, 10, 12 or 16 days were inoculated by various routes using the techniques described by Beveridge and Burnet (1946) and afterwards incubated at 36–37°. After infection of the chorio-allantois the membranes were harvested by cutting through the shell outside the artificial air space, allowing the allantoic fluid to drain away and then removing the inoculated portion, after any excess fluid on the ectodermal surface had run into the removed part of the shell. The chorio-allantoic membrane (C.A.M.) after weighing was ground up with a minimum of sand in a small pestle and mortar, spun at 1500 r.p.m. for 5 min. and the supernatant removed and titrated using Hartley broth as a diluent. By the use of this method of harvesting, any fluid resting on the membrane could be recovered and titrated for virus as well as the membrane. In the case of the growth-curves the C.A. membranes were harvested into bijou bottles, kept at − 35° and all titrated at the same time. Embryo brains were treated after removal in the same way as the C.A. membranes except that an equal amount of Hartley broth was added before emulsifying. Allantoic or amniotic fluids and yolk-sacs were harvested by the technique of Beveridge and Burnet (1946).

Technique of the growth-curves.

The chorio-allantois of each egg was inoculated with 0·05 ml. of virus suspension and 4 eggs were harvested together when desired. The titre of virus was expressed as the number of $I.D._{50}$ doses of virus per membrane as occasionally an embryo died during the experiments and only 3 were left for harvesting at some dilutions. The amount of virus per C.A. membrane was calculated from (1) the total weight of the membranes (T)—1 ml. being considered equivalent to 1 g. for the purpose of the calculation ; (2) the dose inoculated into the mice, *i.e.*, 0·03 ml. ; (3) the number of membranes used (N), and (4) the dilution of the suspension (D) ; *i.e.*, number of $I.D._{50}$ C.A.M.

$$= \frac{T \times D}{0{\cdot}03 \times N}.$$

RESULTS.

Growth of Virus in the Chorio-allantois.

The amount of virus present in the inoculated portion of the chorio-allantoic membrane was determined at intervals after infection with high and low doses of the W.E. strain and with a low dose of the 84B virus. At the same time the virus titre in the embryo brain was noted in four cases. The results are given in Table 1.

In the case of the lower doses (about 1000 $I.D._{50}$ in the mouse) the virus content had fallen at 12 or 24 hr. to 10 per cent or less of the inoculum but increased for the next 24 hr. until a level was reached which was maintained for another 2 days before dropping rapidly to a low level or to zero by the 10th day. Virus did not appear in the brain until between the 2nd and 4th day, rose slowly to a maximum by the 4th to the 6th day and then started to decline, reaching zero in the case of 84B a few days after inoculation.

TABLE I.—*Growth of L.C.M. Virus in the Chorio-allantoic Membrane and Brain of the Developing Chick Embryo after Chorio-allantoic Inoculation.*

Days of incubation of eggs when inoculated	W.E. Strain.						84B Strain.	
	10	12	10	12	12	12	10	10
Tissue titrated	C.A.M.	C.A.M.	Brain.	Brain.	C.A.M.	Brain.	C.A.M.	Brain.
Log of titre of virus of inoculum	4·8	4·8	4·8	4·8	2·9	2·9	3·2	3·2
Log of titre of virus after: 12 hr.	—	—	—	—	1·0	<0·3	—	—
1 day	4·9	4·4	1·0	<0·3	3·0	<0·3	2·1	<0·3
2 days	5·6	5·3	4·2	3·6	4·7	0·9	5·3	<0·3
3 „	—	—	—	—	5·1	3·7	—	—
4 „	5·5	5·8	5·0	4·5	4·9	4·0	5·1	2·2
6 „	5·8	5·5	4·2	4·7	3·3	3·6	4·2	3·0
8 „	5·0	4·7	4·1	4·4	1·2	2·8	2·0	1·4
10 „	4·1	—	3·7	3·9	—	—	<0·4	<0·3

With high doses of the W.E. strain in eggs infected after 10 and 12 days' incubation any drop in titre after inoculation had been restored by 24 hr. and the maximum titre was reached by 48 hr. However, this level was maintained for a longer period and the decline was slower than with the lower dose. The virus in the brain appeared sooner, at 24 hr., increased rapidly at first and then more slowly for 4 days or so. This was followed by a gradual decline, virus still being present to high titre after the chicks had hatched. During the more rapid period of virus multiplication the virus titre increased by between 1,000- and 10,000-fold within 24 hr.

In order to see if the virus of the inoculum could be detected in the chorio-allantois shortly after infection, batches of eggs were titrated at frequent intervals until 12 hr., and then at 24 or 48 hr. to see if growth had taken place. With the

TABLE II.—*Behaviour of the W.E. Strain of L.C.M. shortly after Inoculation of the Chorio-allantoic Membrane.*

Amount of virus stated as log of number of $I.D._{50}$ per membrane.

	Inoculum.			
	2·5.		3·5.	3·1.
Virus in:	C.A.M.	Surface washings from C.A.M.	C.A.M.	C.A.M.
Time after inoculation: 5 min.	<0·3	—	0·3	1·7
10 „	2·1	—	1·5	2·0
20 „	1·2	1·7	—	1·1
40 „	<0·6	—	<0·3	<0·6
80 „	0·9	1·0	—	<0·5
3 hr.	<0·6	—	<0·6	—
5 „	0·8	0·8	—	0·7
12 „	0·7	—	0·4	1·1
24 „	2·4	—	2·1	1·9
48 „	4·0	—	4·9	5·3

three low doses used (Table II), after a small peak at 10 min. the detectable virus fell to a low level (0·21 per cent of the inoculum) and remained at that level for at least 12 hr. before increasing.

In Table II it can also be seen that the virus contents of the drainings from the surface of the membranes had decreased to a very low level by 20 min. showing that

much of the virus had been adsorbed by that time. The lack of infectivity in the membrane was thus not due to delayed virus adsorption.

No pock-like lesions were produced in the chorio-allantois during the growth of L.C.M. virus but the membranes became somewhat oedematous after the third day. Histologically, except for areas of slight infiltration by macrophages and leucocytes into the mesoderm of the oedematous chorio-allantois, no marked changes could be seen.

To determine the distribution of virus in the egg after chorio-allantoic inoculation, six 12-day-old eggs were inoculated with a dose of approximately 3400 $I.D._{50}$ of W.E. virus and harvested 5 days later at which time it was considered that virus should have disseminated widely to embryonic and extra-embryonic tissues. Each of the different fluids and tissues was pooled and titrated for virus.

TABLE III.—*Distribution of L.C.M. Virus in Fertile Eggs after Inoculation by Different Routes with Strain W.E.*

Route of inoculation.	Log of mouse $I.D._{50}$ in inoculum.	Age of developing chick embryo when inoculated.	Days after inoculation when eggs harvested.	Log of number of $I.D._{50}$/g. or ml. of					
				C.A.M.	Brain.	Liver.	Amniotic fluid.	Allantoic fluid.	Yolk-sac.
C.A.M.	3·53	12	5	5·9	5·7	5·4	2·9	2·4	3·2
Amniotic sac	5·8	13	3	—	4·9	—	3·4	<1·0	—
		13	5	—	5·6	—	3·8	<1·0	—
	2·7	13	3	—	3·1	—	<1·0	—	—
		13	5	—	5·1	—	<1·0	—	—
Allantoic sac	3·7	10	3	<1·0	<1·0	—	—	<1·0	—
		10	5	<1·0	<1·0	—	—	<1·0	—
Yolk-sac	3·7	7	5	<1·0	<1·0	—	—	—	3·4

As shown in Table III virus was present in all the embryonic fluids to low titre and in the liver to a titre a little lower than that in either the C.A. membranes or in the embryo brains.

To determine the relative sensitivity of chorio-allantoic inoculation and intracerebral injection of mice, virus was titrated at 10-fold dilutions in both. The C.A. membranes were harvested after 4 days and a 1/20 suspension injected into mice to demonstrate the presence of virus. The result, shown in Table IV, indicated that the mouse, dose for dose, was somewhat more susceptible but that this small difference could be abolished by the larger dose, up to 1·0 ml., that could be used on the chorio-allantois.

No soluble complement-fixing antigen as had been demonstrated in guinea-

TABLE IV.—*Comparison between Infectivity for Mouse Brain and on the C.A.M.*

	Log of $I.D._{50}$ titre in mice (0·03 ml. i.c.).	Log of dilution of virus inoculated on the chorio-allantois of the developing chick embryo giving symptoms on subsequent mouse passage (0·05 ml./C.A.M.).
W.E. strain	4·4	4
84B „	3·8	3

pigs, mice and monkeys by Smadel, Baird and Wall (1939) could be demonstrated in either the C.A., liver or brain of chicks at any time after infection with either high or low virus inocula.

That L.C.M. virus can be passed in eggs indefinitely has been shown by Bengtson and Wooley (1936), Tubaki (1940) and Whitney, Gucil, Lawson and Gordon (1952) but with the two strains used here the time of year influenced the success. Attempts to pass virus in October and early November were not successful. Four passages of the W.E. strain (by the chorio-allantoic route) were carried out without difficulty during April and May. Further passages were not attempted.

To determine the survival of virus in hatched chicks, fifteen 16-day-old embryos were inoculated by the C.A. route with 0·05 ml. of virus suspension containing 10^5 $I.D._{50}$ doses. Thirteen of these hatched out and the brains from pairs of chicks were examined at 2, 4 and 10 days of age for virus. One of the 10-day chicks died, as did 2 others on the 14th and 18th days after hatching. Virus was present in those killed on the 2nd and 4th day but not in those examined on the 10th, 14th and 18th days.

The remaining five chickens were kept for 8 weeks, bled and their serum examined for neutralising and complement-fixing antibodies. Neither was found.

Inoculation of the Developing Chick Embryo by Other Routes.

Groups of 8 fertile eggs were inoculated by different routes with W.E. virus and harvested at 3 and 5 days except for yolk-sac inoculation when all were incubated for 5 days. Tissues or fluids harvested on each day were pooled before titrating virus.

As can be seen from Table III only after amniotic inoculation did virus spread to other parts of the embryo. The virus titre in the amniotic fluid never rose to a high level even after a large dose, but virus was absorbed and appeared in the embryo brain.

After allantoic and yolk-sac inoculation virus did not spread to the embryo and did not appear to multiply in either of these sites although some virus was still present in the yolk-sac after 5 days.

DISCUSSION.

From these results it appeared that the 2 strains of lymphocytic choriomeningitis virus used in these experiments could grow and multiply in the chick embryo after inoculation either by the C.A. or amniotic route but not after inoculation into the allantoic cavity or the yolk-sac.

After chorio-allantoic inoculation the major proportion of the virus inoculum was adsorbed by the surface cells within half an hour and only a small amount (10 per cent) could be demonstrated in an infective form in the membranes. Thus infectious L.C.M. virus disappeared soon after inoculation as has been shown to take place with influenza and other viruses. Multiplication did not occur until more than 11 hr. later. After that the infectivity increased and reached a maximum within 48 hr. which was independent of the size of the inoculum. Infectious virus could be demonstrated in the chick embryo brain within 24–48 hr. with the W.E. and later with the 84B strain, the maximum titre being attained proportionately later than in the C.A.M. Thereafter the virus concentration

decreased rapidly. Although this virus is not strictly " neurotropic " it has an affinity for nervous tissue and these findings would support the view of Sanders (1953) that the " neurotropic " group behaves similarly to other viral agents in their general pattern of growth. With the large doses the virus could be demonstrated in the brain of the hatched chicks for at least 4 days after hatching but by 10 days of age had been completely eliminated. Although the infected chicks were more sickly than the normal controls no evidence could be produced that the virus was directly lethal for them. The three chicks that died at 10, 14, and 18 days of age had no detectable virus in the brain.

Although no antibody could be demonstrated in the survivors it was possible that neutralising antibody had not yet appeared and complement-fixing antibody, although not shown by the usual complement fixation techniques, might have been demonstrated by using the complement fixation inhibition technique of Karrer, Meyer and Eddie (1950). Bengtson and Wooley (1936) were unable to demonstrate virus in one chicken with a leg deformity which had been allowed to hatch after C.A. inoculation with L.C.M. virus. In their series no hatched chickens died although they appeared to be sickly ; nor were they able to infect 3-day-old chicks by the intracerebral route.

That the amniotic route should be the only other way found of readily infecting the chick embryo was of interest. The respiratory tract appeared to be the route by which infection with the W.E. strain spread from one guinea-pig to another. In some unpublished experiments infection could not be shown to occur *via* the alimentary tract, sexually or to be transmitted by lice. Also in a large proportion of human cases of L.C.M. transient lung changes can be demonstrated radiologically. These findings support the view that the respiratory tract is the natural portal of entry to virus in many of the mammals susceptible to lymphocytic chorio-meningitis infection.

Although no complement-fixing soluble antigen could be demonstrated in the egg material with either strain the findings of Whitney *et al.* (1952) indicate that after repeated passage it would appear in detectable amounts. These workers also found virus multiplied to high titre in the yolk-sac after repeated passage and the results in Table III showed that the mouse-passaged W.E. strain of virus could survive without loss in the yolk-sac for at least 5 days. If on adsorption to cells of the yolk-sac virus disappeared as it did in the chorio-allantoic membranes, this would indicate that the multiplication of this strain had occurred in this site. However, no spread to the embryo was noted.

Although the developing chick embryo is of the same order of sensitivity as the mouse to L.C.M. infection, its use in diagnosis offers no advantages over mouse or guinea-pig inoculation. As no pocks are formed the subsequent injection of mice is necessary for the demonstration of virus. As no detectable complement-fixing antigen is produced during the early passages this cannot be used for indicating virus as can sometimes be done following the primary inoculation of guinea-pigs (Smadel and Wall, 1941). The difference in lethality of these two strains for guinea-pigs could not be correlated with the ease with which they grew on the C.A.M. of fertile eggs.

SUMMARY.

The growth of 2 strains of L.C.M. virus in the chorio-allantois of the developing chick embryo has been followed. As with other viruses four phases of growth were

found. First a phase of disappearance of infective virus, secondly a phase of rapid multiplication, thirdly a phase during which the virus titre remained constant and fourthly a stage of decline. The length of survival of virus in the chicks after hatching and the efficiency of different routes of inoculation were also determined. The relation of the findings to virus growth and to L.C.M. infection is discussed briefly.

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OBSERVATIONS ON THE FORMATION AND NATURE OF RUSSELL BODIES.

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THE term Russell body is used in this paper to denote certain intracellular acidophil hyaline bodies, which have been produced experimentally in animals by the injection of bacteria, and which have similar appearances and staining reactions to the structures first recognized by William Russell of Edinburgh and described by him as " the characteristic organism of cancer."

Russell (1890) found examples in a large number of malignant growths of human origin as a feature of the small round-cell infiltration at the margin. Russell concluded that these bodies were an essential component of the neoplastic process, unrelated to degeneration or inflammation of tissue secondary to such neoplastic change. He recorded two apparent exceptions to this rule, a granulomatous ulcer of the leg and an ulcer of the nasal cavity, but he interpreted his sections as showing early signs of the secondary development of a neoplastic change and concluded that the acidophil bodies (fungus particles) were the agent causing this change.

However, the literature contains ample references to the regular presence of Russell bodies in granulomas from a wide variety of chronic infections. An early group of reports described their presence in rhinoscleroma; Pellizzari (1883), Cornil and Alvarez (1885), Konstantinowitsch (1902) and Marschalko (1900). The literature contains numerous accounts of their presence in chronic inflammatory lesions, brain abscesses, syphilis of the central nervous system, skin diseases, syphilitic condylomata, and inflammatory reactions surrounding gastric ulcer.

Russell bodies are also found in small numbers in the " normal " intestinal mucosa of man. Lisco (1942) demonstrated their striking localisation within lymph follicles of the intestinal tract of pigs. Remarkably large numbers occur in the abomasum or fourth stomach of ruminants (Weill, 1919) where they are referred to by this author as *Schollenleukocyten*. Russell bodies are also not uncommon in the abnormal plasma cells of plasmacytoma (Apitz, 1937).

In contrast with the very extensive literature relating to the occurrence of these structures in human and veterinary material is the paucity of reference to their occurrence in the experimental animal. Miller (1931) injected rabbits repeatedly by the intraperitoneal route with a variety of protein fractions derived from the tubercle bacillus. Plasma cells resulted in the omentum and in reticular tissues, and at some of these sites contained eosinophilic bodies (Russell bodies).

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They were thought to represent a degenerative change in the cytoplasm of the mature plasma cells. It was noticed that Russell bodies were numerous where plasma cells were crowded and crowding was suggested as a possible cause of the cytoplasmic degeneration.

The first part of this paper concerns the cellular reactions in reticular tissues following the intravenous injection of a variety of antigens, in relation to the production of Russell bodies. The second part deals with attempts to characterise these structures by histochemical and immunological methods.

MATERIALS AND METHODS.

Vaccines and antigen solutions.

Proteus vulgaris *and* Klebsiella pneumoniae.—The organisms were grown in a tryptic digest broth for 18 hr. at 37°. It was found that this constantly gave opacities approximately equal to that of tube 1 " Wellcome " opacity tubes or 2×10^8 organisms per ml. (Wright method).

Mycobacterium tuberculosis.—A culture of avirulent human bacilli derived from the strain H37Rv was provided by Dr. F. C. O. Valentine. This was grown for 10 days in Dubos medium with added human serum albumin and Tween 80 (Sorbitan mono-oleate). The organisms were heat-killed, thrice washed in physiological saline and reconstituted to a volume half that of the original culture.

Bovine γ-globulin.—Supplied by Armour & Co. Ltd., Lindsey Street, London, E.C.1.

Staphylococcal toxoid (strength B).—Kindly provided by Dr. C. L. Oakley, The Wellcome Research Laboratories, Beckenham, Kent.

Streptococcus pneumoniae.—Type III organisms were provided by the National Collection of Type Cultures. Cocci from the peritoneal cavity of a moribund mouse were grown overnight (14 hr.) on blood agar and washed off into sterile saline. The suspension was standardized to an opacity equal to that of tube 2 " Wellcome " opacity tubes or 6×10^8 organisms per ml. (Wright method). The intravenous dose in the rabbit was 1 ml.

Preparation of frozen sections.

Small blocks of tissue, up to 5 mm., were obtained immediately after death and placed in thin-walled test-tubes, closed with rubber stoppers and quickly frozen by rapid immersion in alcohol-CO_2 mixtures at − 70°. Frozen sections at 4μ were prepared from these tissues by the modification of the Linderstrøm-Lang and Mogensen technique (1938) described by Coons, Leduc and Kaplan (1951). Subsequent handling of sections followed the methods of these authors except that gelatinized slides were not used. The sections were fixed in 95 per cent ethanol for 15 min. at 37° for the demonstration of antigen and antibody, and in Bouin's fixative for staining by Giemsa's method.

Demonstration of antigen and antibody in tissues.

P. vulgaris antigens in the frozen tissue sections were demonstrated by the technique of Coons and Kaplan (1950). Briefly, the procedure (A) for staining of antigen involves a precipitation of fluorescein-tagged *Proteus* antibody, when a solution of the latter is layered over the tissue section and allowed to react for 30 min. The ability of *P. vulgaris* antibody conjugate to reveal homologous antigens in tissues was demonstrated by the use of sections from a mouse injected 10 min. before death with living organisms by the intravenous route (Fig. 8). Staining for antibody (B) was by the method described by Coons, Leduc and Connolly (1953), appropriately modified for *P. vulgaris*. The procedure involved treatment of the frozen sections with a dilute solution of *P. vulgaris* antigen for 30 min. to allow precipitation between antigen and specific antibody in the section. The antigen which was thus precipitated was then itself localised with fluorescein-tagged antibody as above (A), after washing in saline for 5 min. to remove excess of unfixed antigen. Staining according to (B) will reveal both antigen and antibody, and thus sections stained by both methods are necessary to determine that part of the staining in (B) which is due to specific antibody.

In all cases therefore sections were stained by procedures (A) and (B). Further, procedure (A) will also indicate any non-specific staining associated with the use of the antibody con-

jugate solution. Another control for the staining of antibody consisted in the use of heterologous systems, *e.g.*, ovalbumin and fluorescein conjugated homologous antibody, to determine that the first stage of procedure (B) is not a non-specific fixation of antigenic protein.

Antigenic fractions of P. vulgaris.

The organisms were grown for 5 days in 12 l. of digest broth (a modification of Hartley's digest broth as prepared in bulk at the Department of Bacteriology, Harvard Medical School) and separated in a Sharples continuous flow centrifuge. They were next disintegrated in a Booth-Green ball mill (Cambridge Instrument Company Ltd.) and the saline washings from this yielded on centrifugation an easily delimited thick layer of disintegrated organisms between the pearly supernatant fluid and the intact bacilli below. This intermediate layer was carefully removed and lyophilized. The dried, light-brown powder was stored in a desiccator over anhydrous $CaCl_2$ at 4° until required for use.

The antigen solution used in the staining technique above was prepared by extracting 50 mg. of the dried disintegrated bacilli with 5 ml. of phosphate saline buffer (0·15 M-NaCl and 0·01 M-phosphate pH 7·0) at 4° for 2 hr. The suspension was centrifuged at 20,000 r.p.m. for 2 hr. in a refrigerated centrifuge at 4°. The supernatant was taken off carefully with a pipette, heated at 56° for 20 min. and merthiolate (sodium ethyl mercuri-thiosalicylate 1/100 solution, Eli Lilly & Co. Ltd.) added to a final concentration of 1/10,000. The antigen was used at a 1 in 100 dilution in the staining procedures.

Preparation of P. vulgaris *antisera and fluorescein conjugates.*

P. vulgaris antisera were prepared by repeated intravenous injections of rabbits with increasing amounts of 18-hr. cultures of living organisms grown in digest broth. The titres determined by agglutination with a formolised suspension of *P. vulgaris* diluted to correspond with opacity of 5 (Macfarland scale) were 1 : 20,280 and 1 : 40,560.

The sera from two rabbits were pooled, a crude globulin fraction prepared by precipitation with half-saturated ammonium sulphate, conjugated with fluorescein isocyanate and purified by dialysis (Coons and Kaplan, 1950).

Before use the conjugate was absorbed twice with lyophilized mouse liver powder. This is necessary in order to eliminate certain non-specific staining of mouse and rabbit tissues (Kaplan, Coons and Deane, 1949).

RESULTS.

Production of Russell Bodies in Rabbits.

Injections of a P. vulgaris *vaccine.*

During the course of investigations in which rabbits were injected repeatedly intravenously with a variety of antigenic substances it was noticed that numerous cells containing Russell bodies were present in spleen sections. The first antigen found to have this effect was *P. vulgaris*. A suspension of living organisms from an 18-hr. culture in nutrient broth was employed. The course of intravenous injections was started with 0·1 ml., 0·25 ml., 0·5 ml., 1·0 ml., given every third day and continued with the same time interval at the 1·0 ml. dose level for 1–3 months.

Sections of such an animal autopsied 6 days after the last injection showed great hyperplasia of the red pulp, with broadening of the pulp strands by the presence of closely packed mature and immature plasma cells. There were also prominent groups of macrophages, some of which were multinuclear and of great size, often containing phagocytosed polymorphonuclear leucocytes, nuclear remnants and haemosiderin pigment granules. The perifollicular zone was broadened but the plasma cell elements here were not quite as numerous as in the pulp strands.

Russell bodies were found in the mature plasma cells of such cellular proliferations of the splenic medulla (Fig. 1 and 2). It was characteristic that certain

areas showed large numbers of cells containing Russell bodies, while other areas were free from them, although containing numerous plasma cells. The Russell bodies were present as eosinophilic spherical masses in the cytoplasm of plasma cells. No other cell was found to show such changes in this material. First, tiny round " vacuoles " appeared throughout the basophilic cytoplasm. At this stage the bodies are not eosinophilic. Apparently by a process of aggregation these spherical bodies become larger until the cytoplasm is occupied by five to ten or more rounded eosinophilic spherical masses, flattened where they make contact with each other and separated by thin strands of cytoplasm, which are still strongly basophilic. The nucleus, densely stained with haematoxylin and pyknotic, is displaced to the periphery of the cell and distorted by the eosinophil masses. The cells are at this stage very similar to the cells described and illustrated by Russell (1890) for human tissues.

In some of the animals there were also present lozenge-shaped eosinophilic structures within the cytoplasm of neighbouring plasma cells. These structures appeared to be crystals of the material composing the Russell bodies (Fig. 3). In animals receiving *P. vulgaris*, the characteristic form of these appeared to be an equal-sided lozenge with the acute angle of approximately 60°, although bundles of long, narrow crystals were also found.

Russell bodies were consistently absent from those reticular tissues of the normal rabbits which were examined (liver, spleen and bone marrow). This is contrary to statements in the literature (*e.g.*, Downey, 1938) that Russell bodies appear in the reticular tissues of normal rabbits. If this is so, they must be rare, particularly if a thorough autopsy is done to exclude a natural infection.

Russell bodies have never been seen in the reticular tissues of the rabbit after a single injection of *P. vulgaris* even with a 1 ml. dose of 18-hr. culture.

In the course of investigations planned for a different purpose the tissues were examined of 14 rabbits which had been injected with a course of 4 intravenous

EXPLANATION OF PLATES.

FIG. 1.—Russell bodies in plasma cells of the medulla of rabbit spleen after repeated intravenous injections of *P. vulgaris*. Paraffin section. Haematoxylin and eosin. × 880.

FIG. 2.—Rabbit spleen showing a greatly broadened medullary strand with numerous Russell bodies and a few crystals within plasma cells. Repeated intravenous injections of *P. vulgaris*. Paraffin section. Periodic-acid-Schiff technique. × 430.

FIG. 3.—Rabbit spleen medulla. Crystal formation in plasma cells after repeated intravenous injections of *P. vulgaris*. Paraffin section. Phloxin tartrazine. ×880.

FIG. 4.—Rabbit spleen showing crystals within plasma cells of the medulla after repeated intravenous injections of *Kl. pneumoniae*. Paraffin section. Haematoxylin and eosin. × 1280.

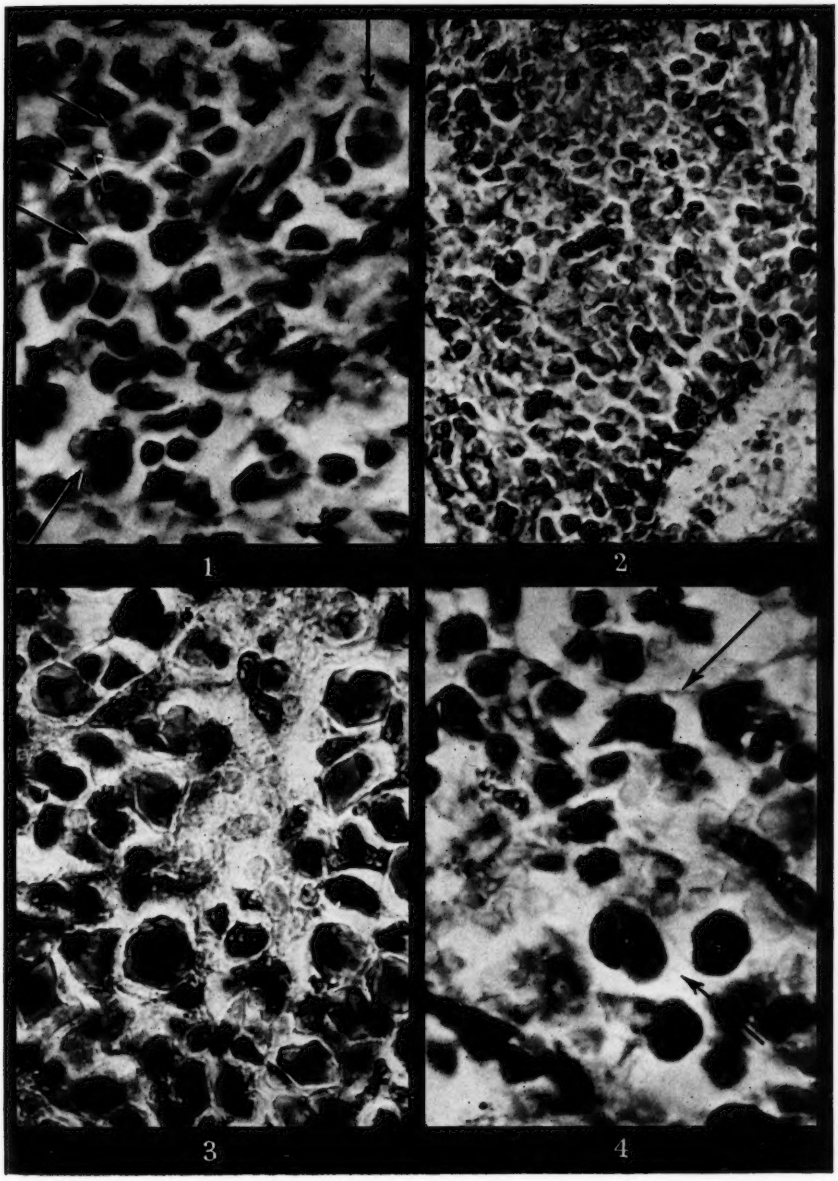
FIG. 5.—Rabbit subcutaneous tissue. Group of mature plasma cells at the edge of a granuloma produced by the injection of diphtheria toxoid adsorbed to aluminium phosphate. Note the stages of development of Russell bodies in the cytoplasm of a group of mature plasma cells (Pc). Eo = Eosinophil. Frozen section. Giemsa. × 900.

FIG. 6.—Fluorescence photomicrograph. Mouse spleen showing numerous cytoplasmic clusters of Russell bodies. The individual Russell bodies are outlined by bright yellow-green surface fluorescence. Frozen section stained for *P. vulgaris* antibody. × 600.

FIG. 7.—Fluorescence photomicrograph. Mouse spleen showing several cytoplasmic clusters of Russell bodies very intensely stained in comparison with the usual antibody-containing plasma cells. Frozen section stained for *P. vulgaris* antibody. × 600.

FIG. 8.—Fluorescence photomicrograph. The light areas represent the bright yellow-green, specifically stained *P. vulgaris* and their disintegration products within Kupffer cells of mouse liver. The liver parenchyma is faintly outlined by its natural blue fluorescence. Frozen section stained for *P. vulgaris* antigen. × 840.

FIG. 9.—Fluorescence photomicrograph. Mouse spleen. Cytoplasmic cluster of Russell bodies. N = cell nucleus. Frozen section stained for *P. vulgaris* antibody. × 1200.



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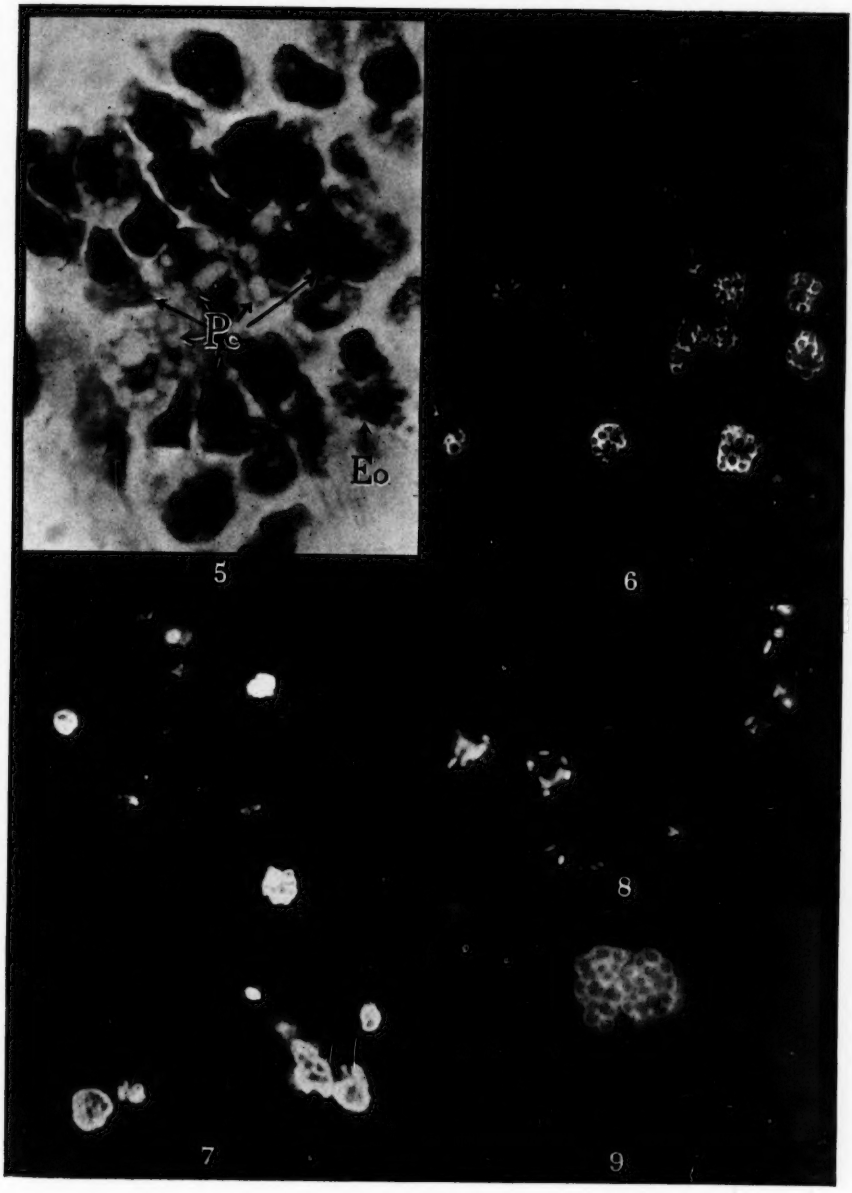

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injections of *P. vulgaris* (an 18-hr. culture in nutrient broth), starting with 0·1 ml. and continuing with 0·25 ml., 0·5 ml., 1·0 ml. at 3-day intervals. With this restricted course of injections, the sections of the reticular tissues do not commonly contain Russell bodies. Occasionally a solitary plasma cell is seen to contain them and in 1 out of the 14 animals a local area of the splenic pulp contained a large number of such cells.

Since, in these experiments, nutrient broth was used, the possible influence of the constituents of this in the production of Russell bodies was checked by the use of organisms which were thrice washed in saline. Two rabbits injected bi-weekly for two months with identical numbers of organisms to those used in broth cultures showed large numbers of Russell bodies in sections of the spleen. Thus it appears unlikely that broth constituents had any influence in the production of Russell bodies.

Experience with organisms other than P. vulgaris.

Russell bodies appeared in the reticular tissues of rabbits when repeated injections of *Kl. pneumoniae* were given intravenously. An 18-hr. culture was used and starting with a dose of 0·1 ml., 0·25 ml., 0·5 ml., and 1 ml. were given with an interval of 3 days, and injections were continued at the 1 ml. level for 5 weeks. Such a course of injections resulted in intense plasma cell proliferation in the reticular tissues with very numerous bodies and crystalline inclusions (Fig. 4).

A similar course of injections of *Str. pneumoniae*, Type III rabbit avirulent, living organisms, was found to produce scattered Russell bodies in the rabbit spleen, although these were far fewer than after courses of *P. vulgaris* and *Kl. pneumoniae*.

The bacillary vaccines used for the production of Russell bodies are unsatisfactory since they consist of complex mixtures of antigenic substances. Accordingly attempts were made with simpler antigens. Intravenous doses of 20 mg. bovine γ-globulin repeated every 3 days for 4 weeks were unsuccessful in two rabbits. Intravenous staphylococcus toxoid 1 ml. dose, repeated every third day for 4 weeks was also unsuccessful (2 rabbits). It should be remarked that both these antigens caused far less impressive plasma cellular accumulations than the bacillary vaccines.

In experiments in which subcutaneous injections of aluminium phosphate precipitated diphtheria toxoid were made into previously immunized animals, Russell bodies were seen in plasma cells in the outer zone of granulation tissue. Fig. 5 shows Russell bodies at varying stages of development in the cytoplasm of a group of mature plasma cells at the edge of such an alum granuloma.

Production of Russell Bodies in Mice.

Experiments were also made in mice. Injections of a suspension of living *P. vulgaris* were given into a tail vein. A course of 0·1 ml., 0·25 ml., 0·5 ml., 0·5 ml. of living organisms from an 18-hr. culture with 3-day intervals between injections and with sacrifice of the animals 6 days after the last, constantly produces in the mouse spleen an intense proliferation of plasma cells. Large numbers of the mature forms of the latter were found to contain Russell bodies. Uninoculated mice from the same source as the experimental animals constantly had small collections of mature and immature plasma cells around arterioles of the pulp, and an occasional one of these contained Russell bodies.

In view of the experiences of Miller (1931) in which fractions from *Myco. tuberculosis* were injected intraperitoneally in rabbits with the production of plasma cells containing Russell bodies, two mice were injected with repeated doses of an avirulent human strain derived from H37Rv. Bi-weekly injections of a suspension of heat-killed organisms were given. The spleen of one mouse autopsied 6 days after the last injection showed very numerous plasma cells with Russell bodies; but in the other animal they were scanty.

Staining and Histochemical Reactions.

The description given below refers to Russell bodies produced in the rabbit by injections of *P. vulgaris*. Paraffin sections were prepared from tissues fixed in formol alcohol. Exceptions to these procedures are stated.

Haematoxylin and eosin.—A noteworthy observation was that the depth of staining with eosin varied. Thus the Russell bodies in the spleen of mice injected intravenously with *Myco. tuberculosis* were intensely eosinophilic. The intracytoplasmic crystalline structures were also stained to a variable degree with eosin.

Haematoxylin-pyronin (Marshall and White, 1950).—The Russell bodies appeared as colourless spherical masses in a network of delicate, intensely pyroninophilic, cytoplasmic strands.

Gram-Weigert stain (Mallory, 1938).—Russell bodies and intracellular crystals were purple when decolorization was such that traces of positive staining remained in collagen.

Phloxin-tartrazine method (Lendrum, 1947).—The larger Russell bodies were strongly phoxinophil but the smaller forms were not stained. The delicate strands of cytoplasm between the bodies were a deep olive-green. The crystals were also phloxinophil.

Giemsa stain.—Frozen sections with subsequent fixation in Bouin's solution. Russell bodies in rabbits were usually a pale pink colour, but those produced in mice by *P. vulgaris* were uncoloured, or pale pink.

Periodic-acid-Schiff (Hotchkiss, 1948).—The details of the technique were taken from Pearse (1949), employing reagents in 70 per cent ethanol. Russell bodies and intracytoplasmic crystals were an intense red-purple. The extended technique as suggested by McManus and Cason (1950) was also used. Acetylation abolished the positive periodic-acid-Schiff reaction of these structures, but treatment with alkali following such acetylation resulted in its restoration. This was regarded as evidence that a chemical structure bearing the 1–2 glycol linkage is a component of such bodies. In some cases control sections were exposed to the action of diastase (saliva) and hyaluronidase (hyalase, Benger) following the technique of Pearse (1953). The P.A.S. reaction was still positive. No evidence was thereby provided that the P.A.S. positive material was either glycogen or hyaluronic acid.

Method for acid polysaccharides (Hale, 1946).—Both the Russell bodies and intracellular crystals gave negative reactions.

Method for arginine-containing proteins (Thomas, 1946).—A weakly positive reaction was given by both the Russell bodies and the intracellular crystals.

Millon's reaction for tyrosine.—A positive reaction (brown-orange coloration) resulted.

Sudan black B.—Staining was by the modification described by Ackerman (1952). No sudanophilia of the Russell bodies could be demonstrated.

In summary Russell bodies produced experimentally as described gave a uniformly positive reaction with periodic-acid-Schiff reagent. This finding taken together with the evidence for the absence of glycolipids, glycogen and acid polysaccharides, suggests that the Russell bodies investigated in this material consisted wholly or partly of mucoprotein or glycoprotein.

The uniformly similar histochemical and staining reactions of Russell bodies and the intracytoplasmic crystals suggested that both are composed of similar material.

Production of Russell Bodies by Injection of Previously Sensitized Rabbits.

In two rabbits the intravenous injection of *P. vulgaris* was continued with two doses of 1 ml. of an 18-hr. broth culture per week for 4 months. The animals were then rested for 6 weeks and at the end of this period a biopsy of the spleen was taken under nembutal and ether anaesthesia and examined histologically. Immediately after the biopsy the animal was injected intravenously with a further 1 ml. and 6 days afterwards was sacrificed. Two controls were injected with a 1 ml. dose of the same culture and sacrificed 6 days later and another two were subjected to biopsy of the spleen before receiving the same intravenous dose of culture. They were also sacrificed 6 days later. These controls were used to show that a single injection with or without the operation on the spleen failed to produce Russell bodies.

The biopsies taken before the recall injection showed a complete absence of the plasma cell reaction, and no Russell bodies were seen. Spleens from the rabbits 6 days after the recall injection showed greatly broadened pulp strands packed with immature plasma cells and lesser numbers of haemocytoblasts and activated reticular cells. The sinuses also contained many immature plasma cells. Also present within the pulp strands were groups of eosinophils and eosinophil myelocytes, as well as scattered pseudo-eosinophils. Russell bodies were very numerous within the plasma cells, of which approximately 10 per cent contained them. Some of these plasma cells contained eosinophil crystals.

To summarize, in rabbits previously sensitized by repeated injections of *P. vulgaris* and rested to allow reversion to a normal splenic histology, the injection of a booster dose of organisms results, 6 days afterwards, in the production of striking numbers of Russell bodies within the plasma cells in the medulla of the spleen.

Time Relations in the Formation of Russell Bodies in Response to a Recall Injection of P. vulgaris.

For economy in animals and material mice were used, and 30 were given a course of intravenous injections of an 18-hr. culture of living *P. vulgaris*. Starting with doses of 0·1 ml., 0·25 ml., 0·25 ml., and 0·5 ml., given at 3-day intervals injections were continued with two further doses of 0·5 ml. (a total of 6 injections). All injections were made into the tail vein. The animals were then rested for 5 weeks and at the end of this period 2 animals were sacrificed as controls, while the remainder received 0·5 ml. as a recall injection. Pairs of these animals were sacrificed at 24 hr., 3, 5, 7, 9 and 11 days following this recall injection.

As a further control group, mice were given a single intravenous injection of 0·5 ml. of the culture used for the recall injection above. Pairs of animals

were sacrificed at the same intervals following this injection. Thus the histological findings in these animals represented the primary response to the organism.

In a rough attempt to quantitate the response in the spleens, the number of Russell bodies in 40 fields using a 43 × objective and a 10 × ocular was counted (an area 30 mm.^{2}). Frozen sections were cut at 4μ and stained by Giemsa and by the method for revealing *P. vulgaris* antibody. The count of cells which contained Russell bodies was made with the latter sections using a fluorescence microscope, since it was very simple to pick out the brightly green-fluorescent cells.

The intravenous injection of *P. vulgaris* in the mouse caused a great proliferation of plasma cells in the red pulp of the spleen. Since spleens from control uninjected mice contained numerous plasma cells in Giemsa-stained sections, it could not be inferred that all the plasma cells were formed in response to the antigenic stimulation of the injected organisms. The plasma cell proliferations were more intense in the animals which were previously sensitized and examined after a recall injection. These differences in cellular content of the two groups were dramatically shown in sections treated by the method for revealing *P. vulgaris* antibody. The primary injection resulted in small numbers of antibody-containing cells, appearing as isolated single cells at the third day and increasing thereafter. The secondary injection resulted in the appearance on the third day of scattered groups of 6–12 antibody-containing cells as well as isolated cells. The animals sacrificed on the fifth day following the recall injection showed large " colonies " of a hundred or so antibody-containing cells producing a generalised mottling of the red pulp. No antibody-containing cells were shown in the white pulp. In animals which were sacrificed at 7 days and after, the proliferations in

TABLE.—*Comparison between the Production of Russell Bodies by Injection of* P. vulgaris *into Normal and Previously Sensitized Animals.*

Days after injection of *P. vulgaris*	Primary response (A). Mouse no.	Primary response (A). No. of Russell body-containing plasma cells in 30 mm.^{2} in spleen section.	Recall response (B). Mouse no.	Recall response (B). No. of Russell body-containing plasma cells in 30 mm.^{2} of spleen section.
1	—	—	798	0
	—	—	799	0
3	802	0	800	0
	803	0	801	10
5	809	0	807	2
	810	0	808	6
7	813	1	811	35
	814	203	812	64
9	890	0	815	100
	883	0	816	8
11	889	0	819	20
	893	0	820	40

Controls : Animals 796 and 797 which had received the same course of sensitizing injections of *P. vulgaris* organisms as other animals in group B were sacrificed on the day of the recall injection. Both animals showed absence of Russell bodies from spleen sections.

discrete groups of cells were less obvious and a confluent mass of antibody-containing cells now outlined the whole of the red pulp of the spleen.

The Table summarises the findings in regard to the numbers of Russell body-containing plasma cells in the spleens of these animals. It will be seen that the primary response is not accompanied by Russell body formation except for one animal (814) of the group which responded not only with a marked plasma cell reaction, but also showed large numbers of Russell bodies in its spleen. Since the number of antibody-containing cells which were found in the sections from this animal was far in excess of that expected in a primary response it was considered possible that natural sensitization to the antigenic components of the test organism had been previously acquired. The results with the group of animals which were stimulated by a recall injection suggested that while Russell bodies can be formed as early as the third day their numbers increase to a peak at a later stage of the reaction, *i.e.*, 7–9 days.

Attempts to Characterize Russell Bodies by Immunological Methods for Revealing Specific Antigen and Antibody.

Since Russell bodies appear in the spleen in response to a variety of antigens it was considered possible that their composition might include specific antibody. As a result of the investigations of Bing (1940), Bjørneboe and Gormsen (1943), Fagraeus (1948) and others, the plasma cell has been thought to be active in the secretion of antibody globulin. Thus it was considered appropriate to investigate the antibody content of such structures by the technique for the localization of antibody in frozen tissue sections described by Coons *et al.* (1953), adapted so as to be appropriate for *P. vulgaris*. Since it was also a possibility that the precipitated antibody of such bodies could result from the presence of specific antigen within such plasma cells, the antigen content was also studied by the technique of Coons *et al.* (1951).

Frozen sections at 4μ thickness were obtained from the spleens of the mice used in the previous section. Areas of antigen or antibody in appropriately treated sections (see " Materials and Methods ") were revealed by the deposition at these sites of fluorescein-conjugated antibody and appeared as bright apple-green areas in the fluorescence microscope.

The sections of animals 801, 807, 808, 811, 812, 814, 815, 816, 819, 820, all showed the presence of varying numbers of Russell bodies in Giemsa-stained sections. In corresponding areas of adjacent sections, treated by the method for antibody, Russell body-containing cells appeared as shown in Fig. 6. It is seen that each Russell body is outlined by a brilliant surface reaction characteristic for *P. vulgaris* antibody. The control sections treated to show the presence of *Proteus* antigen failed to demonstrate any in the Russell bodies. In Fig. 7 the striking brilliance of the fluorescence of the Russell-body surfaces is seen and contrasts with the faint cytoplasmic fluorescence of antibody-containing plasma cells. This appeared similar to the appearance described by Coons, Leduc and Connolly (1953), and White, Coons and Connolly (1953) for plasma cells showing a cytoplasmic content of antibody to diphtheria toxoid and ovalbumin antigens, except that the cytoplasmic fluorescence given by *Proteus* antibody was usually more granular.

In interpreting the results of this technique it may be questioned whether a distinction can be drawn between the presence of antibody within the cytoplasmic

strands surrounding the Russell bodies and the presence of antibody on the surface of the latter. Repeated observations strongly favoured the latter interpretation. As can be appreciated from Fig. 6 and 9, the appearances are those of a reaction at the surface of spherical bodies rather than of a stained network with " holes " for the non-fluorescent bodies. Also the gap shown at N in Fig. 9 represented the compressed and distorted nucleus and it is to be observed that fluorescence does not extend along the peripheral side of this as would be expected if the distribution of the fluorescence coincided with the basophilic cytoplasm.

DISCUSSION.

The histological observations which have been described support the idea that Russell bodies occur within the cytoplasm of plasma cells formed in response to a specific antigenic stimulus. It has been shown that Russell bodies may occur in mice and rabbits in response to a variety of antigenic stimuli. This concept would appear to accord well with the reports of the occurrence and distribution of Russell bodies in regions of inflammatory cellular reaction to chronic infections. The evidence is not sufficiently extensive to allow any generalisations concerning the classes of antigenic material which have this effect. The failure with repeated heavy doses of certain antigens which nevertheless are active in stimulating antibody production and proliferation of plasma cells suggests that Russell body production may be limited to certain types of antigen. Physical properties of antibodies vary and it is to be expected that certain among these would be more easily precipitated within the cytoplasm of plasma cells than others.

Interpretation of the results of the technique for revealing the presence of specific antibody in Russell bodies is not without difficulties, due to the limitations involved in the use of a microscopic precipitin reaction between antigen and antibody. Unlike most tissue stains, the reagents consist of macromolecules with limited penetrating power. A mass of antibody might only give a precipitation of homologous antigen at its surface. It must therefore be realized that the results of the technique as employed here do not allow a distinction between a Russell body structure consisting of a solid mass of antibody and one in which antibody is restricted to a surface layer.

The negative results of the technique for revealing *P. vulgaris* antigens are not, of course, regarded as conclusive evidence that these are absent from Russell bodies occurring after injections of this organism. Antigens could be present and undetectable owing to their valencies being completely occupied in combination with antibody. It was in order to explore this possibility that the technique for revealing antigen was modified; first, by omitting any previous fixation of the section with ethanol, and secondly, by allowing interaction with fluorescein conjugated antibody for prolonged periods up to 2 hr. It was thought that substitution of the antibody molecules of the conjugate might thereby occur with antibody within the Russell body. These manoeuvres also failed to reveal the presence of specific antigens as constituents of the Russell bodies.

The reason for the very strongly positive reaction with the periodic acid-Schiff technique is not very obvious on the interpretation of the structure of the Russell bodies as precipitated antibody globulin. In this connection the work of Blix, Tiselius and Svensson (1941) that normal β- and γ-globulins contain carbohydrate may be relevant. In the experience of the author the cytoplasm of

some normal plasma cells also shows a diffuse reaction with the P.A.S. technique. Pearse (1949) described a similar finding in the case of the cells of plasmacytomata.

There can be little doubt that the " crystals " occurring within the cytoplasm of plasma cells are similar in composition to Russell bodies for they give identical staining reactions and occur in the same conditions and within the same groups of plasma cells. In this connection it may be relevant to note that certain antibodies have been obtained *in vitro* in a crystalline state although this is apparently no evidence that they are pure (Pope and Stevens (1953) in regard to diphtheria antitoxin).

Intracellular crystals associated with plasma cells have also been reported from a variety of human pathological sources. Glaus (1917) and Steinmann (1919) described their presence within the abnormal cells of a plasmacytoma. They have also been described in the cells of granulomas. Thus, the structures " *Zigarrenbunde ähnlich* " described by Mibelli (1889) appear to be examples of crystals within plasma cells of a rhinoscleroma. Zettergren (1949) described protein crystals within the plasma cells of a polyp of the ear, and Freifeld (1913) also described similar crystals in the small round cell infiltration at the margin of an adenocarcinoma.

This account of their occurrence in the experimental animal has not included irrevocable evidence that these structures are truly crystalline, and the opinion is based solely on their morphology. Repeated attempts to demonstrate anisotropism of the " crystalline bodies " or Russell bodies have failed. Zettergren (1949) in describing the crystals present within the plasma cells of an aural polyp concluded that since he could not demonstrate them to be anisotropic and since " inclusions sectioned in a plane parallel with one of the surfaces of the inclusions are always quadrate " the inclusions were members of the cubic crystalline system. If this observation were correct it would be extremely unusual for a protein substance to crystallize within this system. Possibly the fixative (formol alcohol) or the dehydration in absolute alcohol is responsible for a disorientating action upon the protein molecules within the crystal lattice. In the present study frozen sections were restricted to mouse tissues. Crystals were never observed in this material and it was thus not possible to study them in sections uninfluenced by fixatives.

SUMMARY.

Cytoplasmic bodies have been observed in the mature plasma cells which occur in the spleen of rabbits and mice after intravenous injection of repeated doses of various bacillary vaccines.

These structures have a close morphological resemblance and common staining reactions with the " fuchsin bodies " described in human anatomical material by William Russell (1890) and commonly referred to as Russell bodies. This term has been adopted for the bodies with this appearance produced experimentally in animals as described in this paper.

Russell bodies can be produced by a single antigenic stimulus (*P. vulgaris* vaccine) providing the animal has been previously sensitized to this antigen. In mice, it was shown that Russell bodies may appear as early as the third day following such a recall stimulus but occur in maximum numbers at the seventh to the ninth day.

Intracytoplasmic crystals may also develop in mature plasma cells in the same experimental conditions as were found to produce Russell bodies. The material composing such intracytoplasmic crystals has identical staining reactions with the material of Russell bodies.

The surface of the Russell bodies produced in the mouse spleen by the intravenous injection of *P. vulgaris* yielded an intense reaction characteristic for antibody to this organism. The findings with this technique are compatible with the view that antibody is a major component of such Russell bodies.

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THE HISTOCHEMISTRY OF MUCOPOLYSACCHARIDES.

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THERE is a fairly large number of methods available for the histological demonstration of mucin. "Mucin," in the tinctorial sense of the word, is covered chemically by the term "acid mucopolysaccharide." Certain substances, sometimes called mucoid, morphologically indistinguishable from stainable mucin, are not demonstrated by the regular techniques. In all likelihood, they are neutral mucopolysaccharides, since they can be made stainable by esterification with sulfuric acid (Bignardi, 1940*a*, *d*, *f*, *g*; Kramer and Windrum, 1953) or by the oxidative creation of carboxylic groups in them (Bignardi, 1940*b*, *c*, *e*).

Some staining reactions for acid mucopolysaccharides are true histochemical reactions while others are purely empirical. All methods for mucin are used interchangeably, and textbooks of microtechnique tacitly assume that they are equivalent so far as their specificity and sensitivity is concerned. The thesis of the present paper is that the efficiency of these methods is similar but not identical and that different patterns of distribution may be obtained by the use of different methods. Evidence for physicochemical differences between types of mucin will also be presented.

MATERIAL AND METHODS.

Paraffin sections of human gastric and intestinal mucosa and of various dog and guinea-pig organs (salivary glands, gastrointestinal mucosa, kidney, liver, etc.) were used. In most cases, the various staining methods were applied to consecutive serial sections, so as to make an exact comparison of patterns of distribution possible.

The following methods were employed:

(1) Metachromatic staining with toluidine blue.

(2) Metachromatic staining with celestin blue. This method, originally recommended by Lendrum (1947), was modified by using a 0·1 per cent solution of the dye in a 0·5 per cent solution of alum. The nuclei were first stained with haematoxylin and the sections were counterstained with celestin blue for 10 to 30 min.

(3) Mucicarmine. The nuclei were stained with haematoxylin and the sections were counterstained with Southgate's (1927) mucicarmine for 30 min.

(4) A colloidal iron stain of the type described by Hale (1946). The sections were placed for 30 min. in a mixture containing equal parts of 1 per cent solution of saccharated iron oxide, U.S.P., and 2 per cent acetic acid. After thorough rinsing in 1 per cent acetic acid the colour was developed with an acidified solution of potassium ferrocyanide. The mixture recommended was considered superior to the formulae proposed by Hale, and by Rinehart and Abul-Haj (1951) on account of its excellent stability. If prepared with distilled water it will remain usable for at least one month. Tap water may cause turbidity and a non-specific background staining.

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(5) Alcian blue. Steedman's method (1950) was modified as follows: 0·5 per cent alcian blue was dissolved in 5 per cent alum and the sections were stained in the solution for 10 to 20 min., rinsed and counter-stained with alum-Kernechtrot (nuclear fast red, Gurr) or alum carmine. This modification resulted in a completely unstained background, with mucin standing out in a brilliant greenish blue.

(6) Aldehyde fuchsin (Gomori, 1950).

(7) Periodic acid-Schiff (PAS) stain. In some experiments an aldehyde-blocking reagent was interposed between the periodic acid and Schiff's reagent.

RESULTS.

Some types of mucopolysaccharide were stained in an almost identical fashion by all methods, while others were not. The following similarities and differences were observed:

(1) Mucin of the goblet cells of the intestine and of the majority of those of the stomach stained in a similar pattern with all methods except aldehyde fuchsin. The latter stained all goblet cells of the colon but only a minority of those of the stomach. Some goblet cells which appeared to be completely filled with mucin by the other techniques contained only a small amount of aldehyde fuchsin-positive material.

(2) Surface mucin of the human gastric glands stained intensely with the colloidal iron method, much less intensely with mucicarmine and very poorly or not at all with toluidine blue, celestin blue and alcian blue (Fig. 1, 2 and 3).

(3) The bulk of Brunner's glands stained rather well with mucicarmine but remained almost unstained by the other methods, except for scattered cells which were darkly stained.

(4) In the mucous neck cells of the dog's stomach, mucicarmine stained the entire cytoplasm while colloidal iron stained mainly the surface mucus.

(5) In the parotid gland of the dog, scattered mucinous alveoli showed an intense metachromasia but only a very weak staining with colloidal iron and a barely visible one with mucicarmine (Fig. 4, 5 and 6).

(6) The demilunes in the dog's submaxillary glands stained much more intensely than the mucinous acini proper with celestin blue and with mucicarmine, and the reverse held true for colloidal iron. Aldehyde fuchsin stained only the acini; the demilunes remained entirely unstained (Fig. 7).

(7) The unidentified polysaccharide of connective tissue (often referred to as hyaluronic acid) showed widespread, rather marked, although somewhat variable metachromasia with toluidine blue. With the other methods, staining was limited to irregularly scattered areas. For instance, the connective tissue of the renal pyramids stained quite intensely with colloidal iron but was barely recognizable with celestin blue or mucicarmine.

(8) Cartilage matrix stained intensely and uniformly with all methods used.

(9) The mast cells stood out in a brilliant metachromatic shade when stained with toluidine blue; they were stained a slightly purplish blue-black by celestin blue; colloidal iron and alcian blue stained them fairly intensely. They were unstained by mucicarmine.

In addition to these simple staining procedures a few experiments of a histochemical nature were also performed.

Neutral polysaccharides such as glycogen and the neutral mucin of gastric glands can be esterified by concentrated sulfuric acid, as first shown by Bignardi (1940*a*, *d*, *f*, *g*) whose findings have been confirmed by Kramer and Windrum (1953).

In the present experiments it was found that sulfurylation can be performed to great advantage with a 20 per cent solution of concentrated sulfuric acid in anhydrous ether (10 ml. of concentrated sulfuric acid is gradually poured into 40 ml. of ice-cooled ether, and the mixture is stirred carefully). The slides are transferred from alcohol into the sulfurylating mixture and left in it for about 90 min. at room temperature. After a short rinse in alcohol, the slides are washed under the tap. The tissues suffer no damage even if left in the sulfurylating mixture for 24 hr. After this treatment, glycogen, all kinds of mucus and most of the connective tissue stain brilliantly with all the methods mentioned (Fig. 8 and 9). It was interesting to note that the PAS staining of the tissues was not significantly weakened, which indicates that the hydroxyl groups in positions $C_{(2)}$ and $C_{(3)}$ were not esterified by the treatment.

It need not be emphasized that the regular PAS stain showed a much wider distribution of reactive material than any one of other six methods since it stained all periodate-oxidizable polysaccharides, whether acid or neutral. When aldehyde-blocking agents were introduced between periodic acid and Schiff's reagent, highly interesting results were produced. The blocking agents used were the following: cyanide (1 per cent KCN buffered to pH 6·8); dimedon (2 per cent in 50 per cent alcohol); aniline; hydroxylamine and phenylhydrazine (2 per cent solutions in an acetate buffer of pH 4·25). The tissues were exposed to these agents for 4 hr. at 37°. On a theoretical basis, one may have expected that all the aldehydes produced by periodate oxidation would have become non-reactive, giving no colour when Schiff's reagent was subsequently applied. This, however, was not the case. The staining of glycogen and of connective tissue polysaccharides was abolished by all the blocking agents used, but other types of polysaccharides appeared to be more resistant to blockade. Cyanide was the only agent which abolished the reaction completely at all sites. Aniline almost completely and uniformly blocked the reaction. The demilunes of the submaxillary gland were more completely blocked than the acini. Dimedon reduced the staining of mucins only moderately, although it suppressed that of neutral polysaccharides in the serous acini completely (Fig. 10 and 11). Hydroxylamine blocked all mucin-like substances markedly but not completely. In the dog parotid, the mucinous and serous acini showed a pale pink reaction of about the same intensity; in the submaxillary gland the demilunes were darker than the acini. Phenylhydrazine almost completely suppressed all staining except that of the demilunes of the submaxillary gland; they stood out in a brilliant red shade against a very pale pink background (Fig. 12).

DISCUSSION.

These findings of the unpredictable reactivity of aldehydes obtained from carbohydrates by periodate oxidation supplement previous observations on the behaviour of aldehydes derived from nucleic acids (Lessler, 1951*a*, *b*) and of unidentified carbonyl compounds of lipid nature (Gomori, 1952). While these cannot be explained in terms of known facts about the chemistry of the carbonyl compounds concerned, they certainly indicate important differences in their steric structure. The marked dependence of the reactivity of carbonyl compounds on their steric configuration is well known (Stewart, 1907; Michael, 1919). Moreover, carbonyl reactions are, in any case, rarely quantitative, and their

efficiency covers a wide range. For this reason, results of blocking experiments must be interpreted with great caution. More specifically, the failure to effect blockade of a substance with one or even several aldehyde reagents cannot be construed as a clear evidence for its non-aldehydic nature.

SUMMARY.

The tinctorial behaviour of acid mucopolysaccharides of animal tissues exhibits a variable pattern. The reactivity of aldehydes obtained from various polysaccharides by oxidation with periodate is unpredictable.

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DESCRIPTION OF PLATES.

FIG. 1, 2 and 3 ; 4, 5 and 6, and 8 and 9 represent consecutive serial sections.

FIG. 1.—Antral portion of the human stomach ; celestin blue stain. The goblet cells are intensely metachromatic. Contents of glandular lumina barely indicated.

FIG. 2.—The same area ; mucicarmine stain. Goblet cells well stained ; in addition, some staining of glandular contents and surface mucin.

FIG. 3.—The same area ; colloidal iron stain. Goblet cells and mucin within the glands stain with the same intensity.

FIG. 4.—A mucinous acinus in the parotid of the dog ; celestin blue stain.

FIG. 5.—The same area ; mucicarmine stain.

FIG. 6.—The same area ; colloidal iron stain.

FIG. 7.—Submaxillary gland of the dog. Aldehyde fuchsin-mucicarmine stain. The acini proper, stained with aldehyde fuchsin, are black ; the demilunes, stained with mucicarmine, are gray.

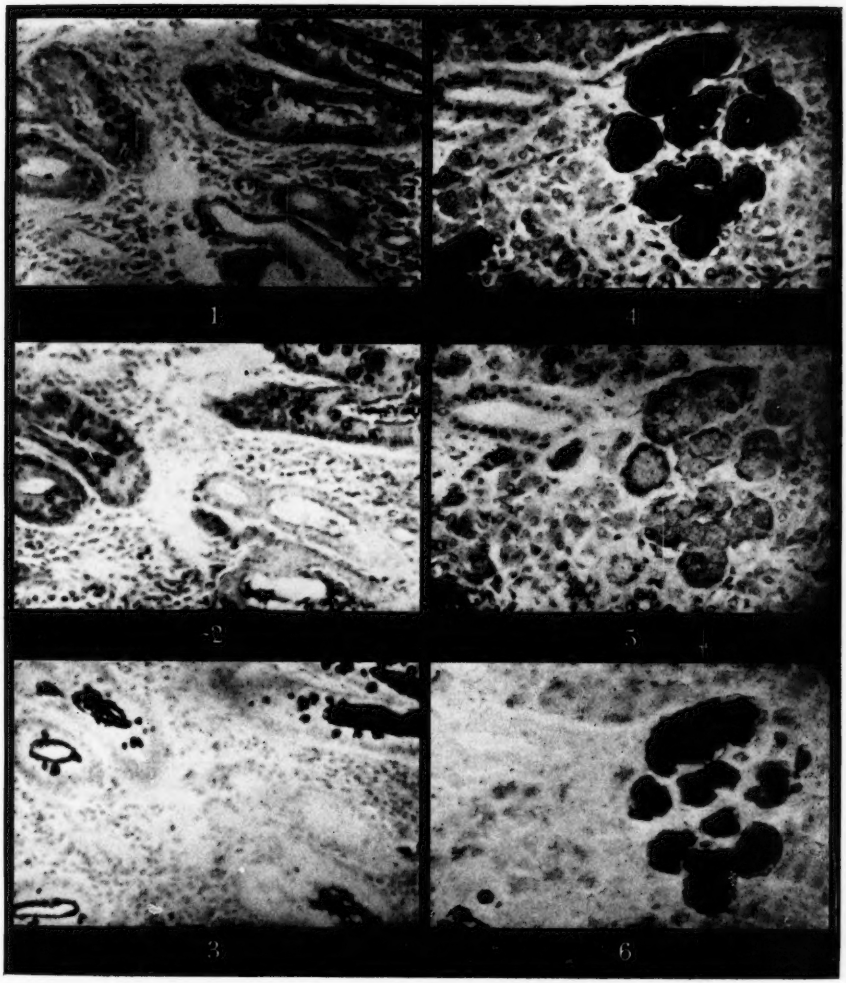
FIG. 8.—Antral portion of the human stomach ; alcian blue stain. A few goblet cells are seen near the upper left corner.

FIG. 9.—The same area. Sulfurylation followed by alcian blue stain. Intense staining of all mucinous structures.

FIG. 10.—A mucinous acinus in the parotid of the dog ; PAS stain. Intense reaction in both the mucinous and serous acini.

FIG. 11.—A different area of the same gland. Periodic acid, dimedon blockade, followed by Schiff's reagent. Mucinous acinus stains intensely ; reaction of serous acini abolished.

FIG. 12.—Submaxillary gland of the dog. Periodic acid, phenylhydrazine blockade, followed by Schiff's reagent. Demilunes stain intensely ; reaction of acini almost completely abolished.



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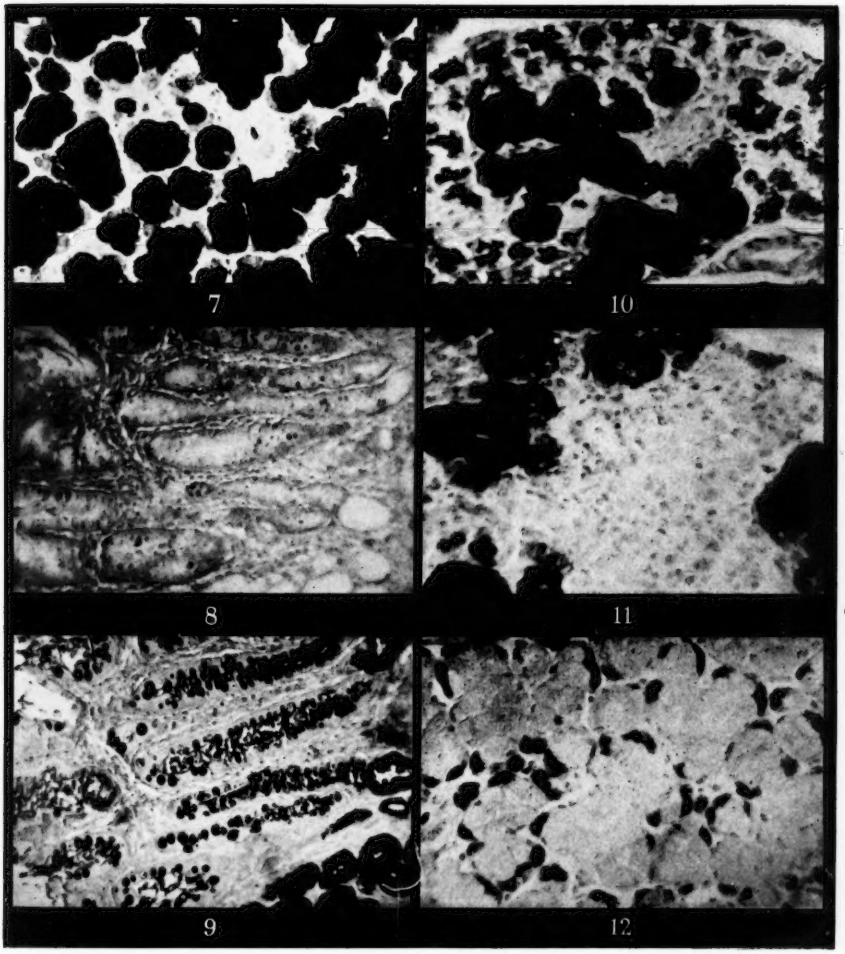
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THE FABRIC OF VIRUS ELEMENTARY BODIES.

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VIRUSES which multiply inside a compliant cell plunder not only its synthetic mechanisms but also, perhaps, its fabric. The conventional view has been that if the complex materials of the fabric of the cell are built into the virus particle, it is only after these substances have been split into relatively simple constituents and have been reassembled to satisfy the particular architecture of the virus. An alternative hypothesis has been proposed by Knight (1946) which suggests that the virus builds its body, in part, from gross fragments of the cell fabric, so that in those instances where a virus is capable of multiplying in the cells of diverse hosts the character of the virus elementary body will be grossly different in each case.

Unfortunately, the evidence to bolster this alternative hypothesis has rested on the serological analysis of virus suspensions ; all the supporting evidence could be easily demolished by asserting that the virus suspension was impure, and was merely mixed with fragments of host-cell protoplasm, either distinct from the virus elementary body, or, at most, adsorbed on to its surface.

A tangible advance in this controversy was made when Smith (1952) suggested that influenza virus purified by adsorption to and elution from erythrocytes might possibly contain host material deep within the particle. This hypothesis was of a very tentative character, but if further work could support it, speculation about the fabric of the virus elementary body would be placed on a much firmer basis.

This paper is concerned with the study of this hypothesis.

MATERIALS AND METHODS.

The serological analyses were all made by complement fixation using the plate technique (Fulton and Dumbell, 1949 ; Le Bouvier, 1953). In estimating the relationship of antigens, standard titres of the antigen were used, defined as that dilution which fixed 0·5 mm.^{3} of complement with excess homologous antiserum. This standardized antigen was used as a constant in a two-dimensional test with ten levels of complement and ten levels of antiserum ; the relationship with the antiserum was expressed as the area under the antigen contour, the scale being 1 cm. for the abscissal units and 3 cm. for the units of the ordinate. The range of experimental error on repetition was rarely more than $\pm$ 5 per cent ; any area which was 5 per cent of the area under comparison or less, was judged not to be significant.

Virus antigen (EB).

Two strains of virus were used, the PR8 strain of influenza A and the Lee strain of influenza B. Eleven-day chick embryos were inoculated intra-allantoically with from 10^{-2} to 10^{-4} dilutions of seed virus ; after 48 hr. they were chilled and the allantoic fluids were

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collected free from blood. To the pooled fluids 2·5 per cent of washed adult fowl erythrocytes were added, and the suspension was kept at 4° for 1 hr. The cells were then deposited by centrifugation and the virus adsorbed to them was eluted for 90 min. at 37° in the veronal buffer used in the complement fixation test. This virus suspension was then purified by another cycle of adsorption and elution, the final volume being one-seventh to one-eighth of the original allantoic fluid.

The antigens had a haemagglutinin titre of 1/2,000–1/10,000 as estimated by a pattern test with a final concentration of 0·25 per cent chick erythrocytes. The antigens were stored in ampoules at − 70°.

In one experiment, noted in the text, a virus antigen was prepared from mouse lung. Mice were infected intranasally with the PR8 strain of influenza A virus which had been passed three times consecutively in mice. After 48 hr. the mice were killed and their lungs were perfused with physiological saline. A 10 per cent (w/v) suspension of the lungs in veronal buffer was prepared by grinding the lungs in a mortar with glass powder. The virus was purified by two cycles of adsorption and elution using guinea-pig erythrocytes. The haemagglutinin titre of the final eluate was 1/640.

Normal chorio-allantoic membrane antigen (CAM).

Chorio-allantoic membranes from 13-day chick embryos were ground in a mortar with glass powder and suspended in veronal buffer (20 per cent w/v). The suspension was centrifuged at 20,000 *g* for 1 hr. The clear supernatant was the antigen and this could be stored for 4–6 weeks at 4°.

This antigen was standardized with serum from guinea-pigs immunized by the intramuscular injection of the antigen.

Normal mouse lung antigen (ML).

Normal mouse lungs, which had not been perfused, were ground in a mortar with glass powder and suspended in veronal buffer (10 per cent w/v). The suspension was centrifuged at 20,000 *g* for 1 hr.: the clear supernatant was the antigen, and this was stored at 4°. It was used at an arbitrary dilution of 1/5.

Immune sera.

Convalescent sera were collected from guinea-pigs infected intranasally two weeks previously. The technique has been described in a previous paper (Ananthanarayan, 1954). Virus antigens, the CAM antigen and the ML antigen were also injected into guinea-pigs and into rabbits. Guinea-pigs received two or four doses of 0·25 ml. intramuscularly at intervals of seven days. One week after the last dose the animals were bled and sera from three to six animals were pooled and stored at − 70°. All guinea-pig sera were inactivated at 56° for 30 min. immediately before use. Rabbits received four doses of 0·5 ml. intravenously at intervals of five days. One week after the last dose the animals were bled and the sera from two rabbits were pooled and stored at − 70°. All rabbit sera were inactivated at 56° for 30 min. immediately before use and then absorbed with 5 per cent sheep erythrocytes to remove natural haemolysin.

Absorption of immune sera.

In some experiments the immune sera were absorbed with normal chorio-allantoic membranes from 11-day chick embryos. The membranes were washed in veronal buffer and then cut by scissors into pieces approximately 2 × 2 mm.; these were suspended in just sufficient veronal buffer to cover them. Equal parts of undiluted inactivated serum and membrane suspension were incubated at 37° for 1 hr.; the membrane was then removed by centrifugation.

Complement.

Pooled guinea-pig serum was stored at − 70°. The different pools did not vary significantly in complement titre.

RESULTS.

In his preliminary work, Smith (1952) had observed in some experiments with purified living influenza A and B viruses that there was no cross complement fixation or a mere trace ; however, when the same preparations were heated and tested with antisera prepared against the heated viruses, there was strong cross reaction. This suggested that there was a common antigen which was deep in the virus structure requiring surface degradation for its exposure. By using control antigens made from extracts of chorio-allantoic membranes from uninfected eggs it was found that most of the cross reaction was due to egg material and anti-egg immune bodies in the virus antiserum. In another context, Bedson (1936) interpreted the changed behaviour of the psittacosis virus antigen after boiling as probably due to the destruction of something on the surface of the virus bodies which unmasked a heat-stable antigen. In an analogous situation Fulton and Begg (1946) have argued that this cannot explain the changed serological behaviour of rickettsiae after boiling. The explanation which they gave to their own observations was that the surface antigen itself had been degraded by the heat and had acquired a new serological specificity.

Smith, Belyavin and Sheffield (1953) admit that it is an open question whether a host antigen is an integral part of the influenza virus elementary body, or is merely adsorbed on to it ; however, their failure to eliminate host antigen by repeated adsorption and elution of virus from red cells incline them to regard it and the virus as inseparable.

In the present work, the serological behaviour of influenza virus preparation, has been compared before and after heating. The experiments to be described support the view that host antigen is a contaminating impurity rather than an integral part of the virus : but it must be admitted that conclusive proof is still wanting.

Convalescent guinea-pig sera.

Guinea-pigs were infected intranasally with live virus and bled after two weeks. In Table I is recorded the reactions of these sera with two virus elementary body antigens which have been subjected to various degrees of heat. The PR8 (EB) preparation, before and after heating, did not react at all with the Lee serum : heating the virus progressively reduced the reaction with the homologous serum.

TABLE I.—*The Effect of Heat on Influenza Virus Antigens Measured by the Complement Fixation Reaction with Convalescent Guinea-pig Sera.*

Antigen.	Treatment.	Area in cm.². PR8 antiserum.	Area in cm.². Lee antiserum.
PR8	Unheated	84·80	0
	50°/15 min.	80·82	0
	56°/15 ,,	42·20	0
	60°/15 ,,	21·13	0
Lee	Unheated	3·00	72·50
	50°/15 min.	5·48	45·35
	56°/15 ,,	1·22	23·10
	60°/15 ,,	1·22	11·88

The Lee EB preparation before heating showed a small reaction with the influenza A serum, but this was below the level of significance ; heating did not materially affect the cross-reaction.

With the homologous serum the Lee virus behaved in the expected manner and showed a progressive decline as the temperature was increased.

Sera from guinea-pigs immunized by intramuscular injection of unheated virus antigens.

If there were impurities in the virus preparations purified by two cycles of adsorption to and elution from fowl erythrocytes, it would have been anticipated that the use of convalescent sera would minimize the risk of inducing antibodies for the contaminating antigens ; whereas intramuscular injection would enhance that risk. From Table II it may be inferred that this has, in fact, occurred.

TABLE II.—*The Effect of Heat on Influenza Virus Antigens Measured by the Complement Fixation Reaction with Sera of Guinea-pigs Immunized by Intramuscular Injection of Unheated Virus Antigens.*

		Area in cm.²	
		PR8	Lee
Antigen.	Treatment.	antiserum.	antiserum.
PR8	Unheated	96·61	13·05
	50°/15 min.	101·06	15·85
	56°/15 „	99·08	17·87
	60°/15 „	76·27	17·87
Lee	Unheated	16·30	105·74
	50°/15 min.	13·01	94·39
	56°/15 „	11·25	89·14
	60°/15 „	10·75	50·30

The guinea-pigs received two intramuscular injections and the cross reactions are now above the level of significance. Heating the virus preparation progressively reduces the reaction with the homologous serum, though less dramatically than when convalescent sera are used. The cross reactions with heterologous sera were in one case very slightly increased, and in the other case very slightly reduced by heating. Absorption of these sera with CAM totally removed the cross reactions with both heated and unheated preparations, but did not materially affect the reactions with the homologous antigens.

Sera from rabbits immunized by the intravenous injection of unheated virus antigens.

Since Smith and his colleagues used rabbit sera, these experiments were repeated with sera from rabbits which had received four intravenous injections of a virus antigen : the results are recorded in Table III. In both cases the cross reactions of strains with heterologous sera were reduced by heating the antigen of the *in vitro* test.

Absorption of these sera with CAM removed the antibodies responsible for the cross reactions, but did not affect the homologous fixation.

The Lee rabbit serum was also shown to fix complement with unheated antigens prepared from a strain of Newcastle disease virus cultivated in the allantoic cavity of chick embryos and purified by two cycles of adsorption to and elution

TABLE III.—*The Effect of Heat on Influenza Virus Antigens Measured by the Complement Fixation Reaction with Sera of Rabbits Immunized by Intravenous Injection of Unheated Virus Antigens.*

Antigen.	Treatment.	Area in cm.². PR8 antiserum.	Area in cm.². Lee antiserum.
PR8	Unheated	75·50	44·90
	50°/15 min.	72·70	42·95
	56°/15 „	63·15	37·35
	60°/15 „	49·50	35·55
Lee	Unheated	41·65	59·80
	50°/15 min.	34·25	50·55
	56°/15 „	29·10	48·95
	60°/15 „	26·70	42·35

from chick erythrocytes. These cross reactions could also be removed by prior absorption of the serum with fragments of CAM.

Sera from rabbits immunized by intravenous injection of heated virus antigens.

Heat degrades the influenza virus progressively; it was found that heating preparations of the virus antigen at 60° for 1 hr. completely destroyed their complement-fixing capacity with homologous convalescent sera.

Accordingly, rabbits were immunized by the intravenous injection of these heated virus antigens and their reactions with the unheated and heated elementary body antigens are recorded in Table IV.

TABLE IV.—*The Effect of Heat on Influenza Virus Antigens Measured by the Complement Fixation Reaction with Sera of Rabbits Immunized by the Intravenous Injection of Heated Virus Antigens.*

Antigen.	Treatment.	Area in cm.². PR8 antiserum.	Area in cm.². Lee antiserum.
PR8	Unheated	17·85	19·40
	60°/1 hr.	17·89	17·85
Lee	Unheated	32·10	31·10
	60°/1 hr.	21·30	29·50

It is notable that heating the virus has very little effect on its capacity to fix complement with these sera; moreover, the reactions with the heterologous strains may be greater than with the homologous even though all the antigens have been standardized for their content of specific heat-labile virus antigens.

The Lee serum was absorbed with CAM, and its reactions with heated and unheated Lee(EB) antigen and with CAM antigen are shown in Table V. The Lee antigens used in this experiment and in the experiment recorded in Table IV are from different batches; although both have been standardized to contain the same amount of heat-labile specific antigen the fixation areas in the two cases are very different. Three absorptions completely remove the antibodies reacting with the unheated antigen and the CAM antigen, and greatly reduce the reactions with the heated antigen.

TABLE V.—*Sera of Rabbits Immunized by the Intravenous Injection of Heated Lee Antigen and Absorbed with CAM : Reactions with Unheated and Heated Lee Antigen and with CAM Antigen.*

Antigen.	Treatment.	Area in cm.². Lee antiserum. Unabsorbed.	Absorbed with CAM—once.	Absorbed with CAM—thrice.
Lee	Unheated	12·35	0·97	0
	60° 1 hr.	18·35	10·68	5·10
CAM*	Unheated	17·75	0	

* CAM = Chorio-allantoic membrane. This antigen was used at an arbitrary dilution of 10^{-2}.

It is inferred that the antiserum contains many antibodies specific for the antigens of chorio-allantoic membrane, and, in addition, a smaller number of antibodies specific for the heated virus antigens ; these latter antigens have a changed serological specificity and antibodies specific for them do not react with the unheated virus antigens.

Antisera prepared against CAM.

All the results obtained so far could be interpreted to mean that virus preparations purified by two cycles of adsorption and elution still contained fragments of chorio-allantoic membrane as an impurity, provided it could be shown that unlike the virus antigens the antigens of CAM were very heat stable. Guinea-pigs were given four intramuscular injections of CAM and the reactions of the sera obtained are recorded in Table VI.

TABLE VI.—*The Effect of Heat on CAM Antigen Measured by the Complement Fixation Reaction with Sera of Guinea-pigs Immunized by the Intramuscular Injection of Unheated CAM Antigen.*

Antigen.	Treatment.	Area in cm.². CAM antiserum.
CAM*	Unheated	46·00
	50°/15 min.	39·18
	56°/15 „	37·75
	60°/15 „	37·28

* CAM = Chorio-allantoic membrane, used at standard titre.

From these results it is clear that the antigens of CAM are very heat stable. Antisera prepared in rabbits by the intravenous injection of CAM behaved in just the same way : indeed, heating the antigen of the *in vitro* test at 60° for 1 hr. often increased the reaction.

It will be observed that in the preparation of antisera against the CAM antigen by the intramuscular injection of guinea-pigs, four injections were given. This was necessary because two injections failed to elicit measurable antibodies. This, however, does not conflict with the supposition that the antibodies present in the sera of guinea-pigs immunized with two intramuscular injections of virus antigen are due to impurities in the virus preparation. For if the CAM antigen is combined with PR8 virus prepared in mouse lung and purified by adsorption to and elution from guinea-pig erythrocytes, two intramuscular injections into guinea-

pigs evoke measurable antibodies againt CAM antigen. Table VII records this experiment.

It is concluded that in the presence of the virus antigen a smaller amount of CAM antigen is effective in provoking antibodies than is required when the CAM is injected alone.

TABLE VII.—*The Antibody Response of Guinea-pigs Receiving Two Intramuscular Injections of PR8 Influenza Virus Grown in Mouse Lung, Compared with the Antibody Response when the Same Virus Antigen was Mixed with a Small Amount of CAM Antigen.*

	Area in cm.².	
Antigen.	Mouse PR8 antiserum.	Mouse PR8 + CAM antiserum.
Mouse PR8 . .	46·10	48·00
Mouse Lung . .	2·51	4·49
CAM* . . .	1·26	10·92

* CAM = Chorio-allantoic membrane. This antigen was used at an arbitrary dilution of 1/3.

The presence of CAM antigen in preparations of virus elementary bodies.

It is easy to demonstrate that virus preparations purified by two cycles of adsorption to and elution from chick erythrocytes react with antisera prepared by the immunization of rabbits with CAM. Moreover, different virus preparations, all standardized to fix the same amount of complement with a convalescent guinea-pig serum fix different amounts of complement with these CAM antisera (Table VIII). This fact argues very strongly against the view that the CAM antigen is an integral part of the virus, for in that case there would be a fixed proportion of such material relative to the virus concentration.

TABLE VIII.—*The Variable Potency of Three Standardized Preparations of Virus Antigen Measured by the Complement Fixation Reaction with Sera of Rabbits Immunized by the Intravenous Injection of Unheated CAM.*

		Area in cm.².
Antigen.	Batch No.	CAM antiserum.
PR8 .	1 .	32·73
	2 .	43·27
	3 .	23·74
CAM* .	1 .	36·92
	2 .	36·75

* CAM = Chorio-allantoic membrane, used at standard titre.

DISCUSSION.

The idea that normal host material is deep inside the influenza virus elementary body was proposed, very tentatively, to explain the observation that in some cases heating of the virus was necessary before cross reactions with heterologous antisera could be demonstrated.

It must be admitted that in the present work it has not been possible to provide conclusive proof that this idea is untrue ; however all the observations recounted

in this paper are consistent with the much simpler hypothesis that the host material is present as an impurity in the virus preparation. Cross reactions could be demonstrated under certain conditions before heating, and although it may be that the degree of purification achieved was inferior to that obtained by other workers, yet no enhancement of the cross reaction was observed after heating.

Nor do these observations support the idea that the host material is built into the surface of the elementary body as an integral part of it. It is possible that some or all of this material is adsorbed to the surface of the elementary body, and so it need not necessarily be removed by many cycles of adsorption and elution.

It has been shown by Smith *et al.* (1953) that the antigen in chick chorio-allantoic membrane is considerably more heat stable than the host antigen in virus preparations. It would be dangerous, however, to draw any far-reaching conclusions from this difference, for the normal CAM antigen is not a perfect control for an infected membrane, and there is no information of the relative strengths of the antigen in the two preparations.

SUMMARY.

Antisera have been prepared against purified suspensions of PR8 and Lee elementary body antigens, in guinea-pigs by intranasal and intramuscular injection, and in rabbits by intravenous inoculation.

The cross reactions between PR8 and Lee virus, heated and unheated, have been studied by the complement fixation reaction. These experiments support the belief that the normal chick antigens, which can be demonstrated in the virus antigens, are present as an impurity; the chick antigen is outside the virus elementary body, probably adsorbed to its surface.

These experiments provide no evidence to support the idea that the host material is an integral part of the virus, deep inside it.

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THE EFFECTS OF ACETYLATION OF AMINO GROUPS ON THE REACTIONS OF ANTIGENS AND ANTIBODIES.

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ONE of the plausible theories advanced to account for the specific combination of antibodies with antigens is that their specific affinity depends on the attraction between oppositely charged groups arranged in complementary patterns on the antigen and antibody molecule. The ionized groups that may be involved are the negatively charged carboxyl groups and the hydroxyl groups of tyrosine on the one hand, and the positively charged amino and guanidinium groups on the other. The imidazole group of histidine can be excluded, as its range of dissociation lies within that in which precipitation is not affected by hydrogen ion concentration.

If the specific affinity depends on NH_3^+ groups it should be lost when these are converted into neutral groups. This is readily effected by treatment with formaldehyde and a large number of studies have been made of the effects of formaldehyde on the specific activities of antigens and antibodies. However, formaldehyde has effects other than that on amino groups; for example, cross linking between protein molecules (Fraenkel-Conrat and Mecham, 1949); so that any effects on the specific activities of antigens and antibodies may be due to changes other than the blocking of NH_3^+ groups.

Each antibody molecule probably carries two receptors for antigens. Supposing that NH_3^+ groups are essential parts of these receptors, it is possible that each receptor contains only one NH_3^+ group. There are about 70 NH_3^+ groups in an antibody molecule; thus unless a large proportion of these are blocked it is improbable that the NH_3^+ groups of both receptors will be affected. If only half the NH_3^+ groups of the molecule are blocked about half of the molecules will have one receptor in which the NH_3^+ group is neutralized and one receptor in which it is not; that is, about half of the antibody molecules will be true univalent antibodies.* In order to get clear-cut results at least 70 per cent of the amino

* The estimate is made as follows:
The total number of ways in which 35 out of 70 groups can be neutralized is:

$$\frac{70 \times 69 \times \ \ldots \ \times 36.}{35\,!}$$

The number of ways in which 35 of the remaining 68 groups can be neutralized when the two groups of the 2 receptors are left unaffected is:

$$\frac{68 \times 67 \times \ \ldots \ \times 34.}{35\,!}$$

The percentage of molecules in which the NH_3^+ groups of the 2 receptors are not affected is:

$$\frac{68 \times 67 \times \ \ldots \ \times 34 \times 35\,!}{70 \times 68 \times \ \ldots \ \times 36 \times 35\,!} \times 100 = \frac{1190}{4830} \times 100 = 24{\cdot}6.$$

(*continued on p.* 390)

groups should be inactivated. It is therefore essential that the treatment used should be one that will not only block most of the NH_3^+ groups but will also not confuse the issue with other reactions.

Ketene has been used to acetylate NH_3^+ groups of antigens and antibodies (Chow and Goebel, 1935; Pappenheimer, 1938; Sandor and Goldie, 1938); but when most of these groups are acetylated the hydroxyl groups of tyrosine may be acetylated as well (Sandor and Goldie, 1938; Olcott and Fraenkel-Conrat, 1947), and in some proteins the hydroxyl groups are acetylated before the NH_2 groups (Li and Kalman, 1946). Ketene is, therefore, not wholly satisfactory. According to Fraenkel-Conrat, Bean and Lineweaver (1949) most of the NH_3^+ groups of proteins can be acetylated with acetic anhydride without affecting the hydroxyl groups of tyrosine. We have studied the effects of acetylation with acetic anhydride on the specific activities of antibodies and antigens. In addition, since Fraenkel-Conrat and Mecham (1949) claim that excess of acetamide, at pH about 4, prevents the cross linking that usually results from treatment of proteins with formaldehyde, we have tried the effect of this method.

As one result of these treatments may be the formation of incomplete antibodies—*i.e.*, antibodies that combine with antigen but do not form precipitates, owing either to inactivation of one receptor (true univalent antibody) or to secondary changes in the structure of the molecule—we have attempted to measure incomplete antibodies by the method first used by Heidelberger and Kendall (1935) and used more extensively by one of us in previous work (Orlans, 1952; 1953).

MATERIALS AND METHODS.

Antibodies and antigens.

Crystalline bovine serum albumin (B.S.A.) and crystalline lysozyme were obtained from Armour & Co. Horse serum albumin (H.S.A.), prepared by Mr. R. G. S. Johns, had been crystallized three times. Ovalbumin, prepared by Hopkins' method, had been crystallized three times. Gamma-globulins were prepared from the rabbit antisera against H.S.A., B.S.A. and lysozyme by a modification of Kekwick's (1940) method (Marrack, Hoch and Johns, 1951). Digested diphtheria antitoxin and purified diphtheria toxin were supplied by Dr. C. G. Pope of the Wellcome Research Laboratories. Horse antiserum against Type I pneumococcus was supplied by the Lister Institute; a Felton globulin solution was prepared from this antiserum by dialysing in the cold against 0·01 M phosphate buffer, pH 5·5; the ppt. was washed with cold buffer and re-dissolved; 42 per cent of the protein of this soln. was precipitable by the polysaccharide. Type I polysaccharide (S I) was prepared by Mr. R. G. S. Johns according to Heidelberger and Scherp (1936). Preparations of globulin,

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The number of ways in which 33 of the remaining 68 groups can be neutralized when the two NH_3^+ groups of the 2 receptors are neutralized is:

$$\frac{68 \times 67 \times \ \ldots \ \times 36.}{33!}$$

$\therefore$ The percentage of molecules in which both the NH_3^+ groups of the 2 receptors are affected is:

$$\frac{68 \times 67 \times \ \ldots \ \times 36 \times 35!}{70 \times 69 \times \ \ldots \ \times 36 \times 33!} = \frac{1190}{4830} = 24{\cdot}6.$$

The percentage of molecules in which the NH_3^+ group of one receptor is blocked and the other is not, is:

$$100 - (24{\cdot}6 + 24{\cdot}6) = 50{\cdot}8.$$

In this calculation it is assumed that all the NH_3^+ groups are equally available to treatment with the reagent. This assumption may be unjustifiable. If amino groups form part of the receptors they must be near the surface of the antibody molecule; but they may lie at the bottom of crypts into which the determinants of the antigen enter. Such NH_3^+ groups would be less accessible than those on the surface but more accessible than those in the interior of the molecule.

consisting mainly of antibody, and a sample of Type III polysaccharide (S III) were given by Dr. J. Humphrey of the National Institute for Medical Research.

Acetylation.

For acetylation the protein soln. was buffered with sodium acetate (about 0·3 g./ml.). Acetic anhydride was added to the ice-cold solution in 120 or 60 portions from an Agla syringe over a period of 1 hr. The solution was kept in ice water and shaken continuously by hand during the addition. Precipitates formed in the soln., particularly in the globulin and lysozyme soln.; this did not interfere with the reaction. After the acetic anhydride had been added the soln. were left for 1 hr. in the cold, dialysed against saline and then neutralized, or dialysed from the first against buffered saline. The acetylated lysozyme was insoluble at neutral pH, but dissolved when diluted and brought to pH 9. Reduction of the pH of this dilute soln. by addition to heterologous rabbit serum did not cause re-precipitation. The other acetylated proteins re-dissolved when dialysed against saline and neutralized.

Antigen–antibody reactions.

The reactions of the treated antigens with untreated antibodies were studied by measuring the ppt. formed by varying amounts of these treated antigens with constant amounts of homologous antisera or γ-globulin solutions.

The maximum antibody precipitated by the untreated and treated antigens were calculated by subtracting from the maximum ppt. the corresponding amount of antigen. Thus in Exp. 2 (Fig. 1) the maximum ppt. with untreated antigen 1·77 mg. was obtained when 0·275 mg. of antigen was added; the maximum antibody precipitated by untreated antigen was $1·77 - 0·275 = 1·495$ mg. Similarly the maximum antibody precipitated by acetylated antigen was $1·20 - 0·197$ mg. $= 1·00$ mg. The per cent of total antibody precipitated by the acetylated antigen was $\frac{1·00}{1·49} \times 100 = 67$.

To study the reactions of the treated antibodies varying amounts of homologous untreated antigens were added to constant amounts of the acetylated antibody globulins. In most cases no ppt. formed during the time of observation or precipitation was slow and incomplete. To measure incomplete antibodies varying amounts of antigen were added to constant amounts of treated antibody globulin solution. After these mixtures had been kept at room temperature for 1 hr. or more, constant amounts of untreated antiserum or antibody globulin soln. were added to each and they were kept at room temp. until floccules appeared and at 4° for not less than 3 days. The ppt. were then measured as usual. The incomplete antibodies were assumed to be co-precipitated with the untreated antibody. As the amount of antibody in the solution of untreated antibody added was known, the co-precipitated protein could be estimated by difference.

If acetylation had caused aggregation of antibody with other molecules in the globulin soln., this other globulin would also be co-precipitated; in that case the co-precipitated protein would exceed the actual antibody in the treated γ-globulin soln. The co-precipitated protein is therefore called apparent antibody.

The antibody soln. was diluted in the course of acetylation, dialysis and neutralization. The amount of antibody which would be present in the acetylated soln., if it was not affected by the acetylation, was calculated on the assumption that the ratio of antibody to total protein in the soln. remained unaltered.

The following is an example of the calculation:

Exp. 7 (Fig. 3). The original γ-globulin soln. contained 3·0 per cent of protein and 7·3 mg. of antibody per ml. The protein concn. after acetylation, dialysis and neutralization was 1·49 per cent; the amount of antibody that would be present, if the ratio of antibody to total protein remained unaltered, would be $\frac{1·49}{3·00} \times 3·65 = 1·81$ mg. per 0·5 ml. The amount of untreated antibody added was 0·28 mg. The apparent antibody precipitated, calculated from the upper curve, Fig. 3, was 1·53 mg. per 0·5 ml.; subtracting the 0·28 mg. of untreated antibody added, we find that the apparent antibody derived from the acetylated γ-globulin was 1·25 mg. per 0·5 ml. The apparent antibody recovered was $\frac{1·25}{1·81} \times 100 =$ 69 per cent.

The amount of protein in the ppt. was estimated by micro-Kjeldahl and by the method of Folin and Ciocalteu, modified by Heidelberger and MacPherson (1943). When the treated antibody did not give the full colour with the phenol reagent, allowance was made for this alteration in the estimation of the ppt. The factor 6·25 was used in calculation of protein from nitrogen determinations. As the nitrogen content of lysozyme is high, the results of experiments with this antigen were expressed in terms of nitrogen.

The amino groups of untreated and treated proteins were estimated by the formaldehyde method of Kekwick and Cannan (1936); in Exp. 5 and the experiment with formaldehyde and acetamide by the ninhydrin method of Miller and Stanley (1941). Residual tyrosyl residues were measured by the Folin-Ciocalteu method; no evidence of reduction of these residues was found in the acetylated proteins. Dr. Beaven of the Medical Research Council Spectrographic Unit, London Hospital, plotted the ultraviolet absorption curve of one of the samples of acetylated B.S.A. (Exp. 2) and found no evidence of acetylation of the tyrosyl residues.

RESULTS.

Acetylated Antigens.

The results of acetylation of antigens are shown in Table I.

TABLE I.—*Degree of Acetylation of Amino Groups of Antigens and Per Cent of Untreated Antibody Precipitated by the Acetylated Antigen from Homologous Antisera.*

Experiment No.	Antigen.	Weight of antigen acetylated (mg.).	Acetic anhydride used (ml.).	Per cent amino groups blocked.	Per cent of antibody precipitated.
1	B.S.A.	250	0·5	90	74
2	B.S.A. (Fig. 1)	250	0·5	Over 90	67
3	Ovalbumin	56	0·12	Over 90	75
4	Lysozyme (Fig. 2)	230	0·46	75	86

In Exp. 2 only 67 per cent of the antibody was precipitated by acetylated B.S.A. To test whether acetylated B.S.A. combines with the residual 33 per cent of antibody that was not precipitated by it, an experiment was made to find out whether excess of acetylated B.S.A. would inhibit ppt. formation with untreated B.S.A.:

To 1 ml. of 1/4 anti-B.S.A. serum, that contained 3·6 mg. of antibody, 2·7 mg. of acetylated B.S.A. was added; this was about eight times the amount that would give maximum precipitation with this amount of antiserum. After the mixture had been kept at room temp. for 50 min. 0·075 mg. of untreated B.S.A. was added and the mixture was kept at 4° for 5 days; 0·54 mg. of ppt. formed. With an amount of antibody equal to 33 per cent of the antibody used, 0·075 mg. of B.S.A. should form 0·9 mg. of ppt. The formation of ppt. by untreated antigen and residual antibody was only partially inhibited by excess of acetylated B.S.A.

In order to determine the form of the precipitation curve given by the residual 33 per cent of antibody with untreated B.S.A., 4 ml. of γ-globulin soln., containing 11·2 mg. of anti-B.S.A. antibody, were treated with 1·39 mg. of acetylated B.S.A. This was the amount of antigen required to give maximum ppt. with the γ-globulin soln. The mixture was kept for 3 hr. at room temp. and for 3 days at 4°; the ppt. was centrifuged down. Varying amounts of untreated B.S.A. were added to aliquots of the supernatant fluid and the ppt. were measured. The curve is shown in Fig. 1.

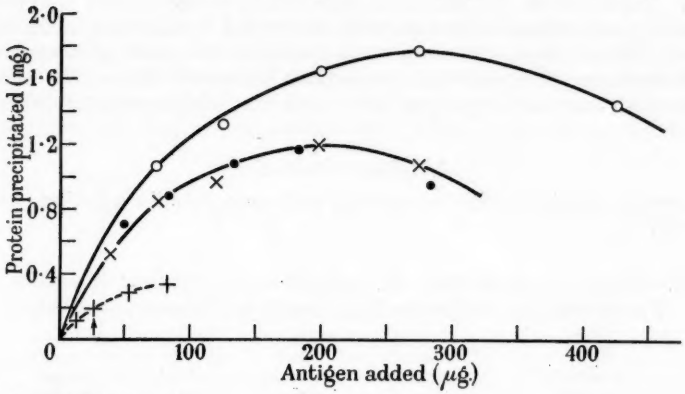

FIG. 1.—Precipitation curves:

○—○ Untreated B.S.A. with 0·5 ml. γ-globulin solution containing anti-B.S.A. antibody.
×—× Acetylated B.S.A. with 0·5 ml. of the same γ-globulin solution.
●—● Untreated B.S.A. with 0·33 ml. of γ-globulin solution, calculated from the —○—○— curve for comparison with —×—×—.
+—+ Untreated B.S.A. with supernatant after removal of antibody precipitable with acetylated B.S.A.; aliquots of supernatant equivalent to 0·5 ml. of original γ-globulin solution.

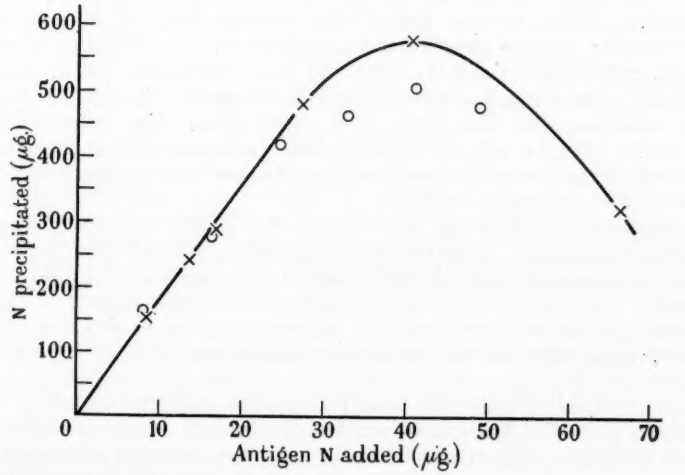

FIG. 2.—Precipitation curves;

×—× Untreated lysozyme with 0·5 ml. antiserum.
○—○ Acetylated lysozyme with 0·5 ml. antiserum.

In all cases antisera flocculated more slowly with acetylated than with untreated antigens. Thus in Exp. 2 (Table I) an anti-B.S.A. serum diluted 1/40 flocculated at optimum proportions in 30 min. with untreated B.S.A. and in 90 min. with acetylated B.S.A. But with acetylated lysozyme the time of flocculation was increased much less; at optimum proportions antiserum diluted 1/50 flocculated in 50 min. with untreated lysozyme and in 90 min. with acetylated lysozyme.

Acetylated Antibodies.

The results obtained with acetylated antibody-globulin solutions are shown in Table II.

TABLE II.—*Degree of Acetylation of Antibodies and Per Cent of Initial Antibody Precipitable by Antigen in the Presence of Untreated Antibody.*

Experiment No.	Antibody against.	Globulin acetylated (mg.).	Antibody in globulin (mg.).	Acetic anhydride added (ml.).	Per cent of amino groups blocked.	Per cent apparent antibody recovered.
5	H.S.A.	61·5	21	0·20	40*	96
6	B.S.A.	90	21	0·25	Over 90	87·5
7	B.S.A. (58) (Fig. 3)	285	69·6	0·6	80	70
8	Ovalbumin	75	29·5	0·15	75	67
9	Diphtheria toxin	190	33	0·5	Over 90	50
10	S I	265	110	0·5	Over 90	26
11	S III (Fig. 4)	46	42	0·10	90	53

* Ninhydrin method.

In all but one case the mixtures of acetylated antibody-globulin and antigen flocculated much more slowly than mixtures of untreated antibody with the same antigen. Thus, mixtures of acetylated anti-H.S.A. globulin, Exp. 5 (Table II) with untreated H.S.A. became cloudy after keeping for 1 hr. at room temp. and at 4° overnight; but no ppt. formed in 2 days at 4°. Mixtures of acetylated anti-B.S.A. γ-globulin (Table II, Exp. 7) with untreated B.S.A. were cloudy after 1 hr. at room temp. and ppt. formed in the cold. The ppt. were measured after the mixtures had been kept at 4° for 6 days. The precipitation curve (Fig. 3) shows that 0·9 mg. of antibody was precipitated directly with antigen from 0·5 ml. of the acetylated γ-globulin, whereas 1·25 mg. per 0·5 ml. was co-precipitated with untreated antibody.

A precipitate formed slowly in mixtures of S I with acetylated Type I anti-pneumococcal globulin. After the mixtures had been kept at 4° for 5 days the maximum apparent antibody precipitated was 18 per cent of the original antibody.

However acetylated anti-S III globulin flocculated rapidly with antigen. The amount of antibody precipitated, measured as usual, was 1·6 mg. per 0·5 ml.—that is, the same as the amount co-precipitated with untreated antibody (Fig. 4).

In Exp. 6 (Table II) the amount of untreated antibody added proved to be rather more than the amount of apparent antibody co-precipitated from the acetylated globulin. The same amount of apparent antibody was co-precipitated when the amount of untreated antibody was reduced to $\frac{1}{3}$ of the co-precipitated antibody. The amount of acetylated antibody co-precipitated was independent of the amount of untreated antibody added.

Formaldehyde-acetamide-antibody.

Anti-H.S.A.-γ-globulin.—Acetamide (100 mg.) was dissolved in 4 ml. γ-globulin soln. containing 11 mg. anti-H.S.A. antibody, and N-acetate buffer was added to bring the pH to 4·0; 0·8 ml. of Analar formaldehyde soln. was added and the mixture left at room temp. for 2 days. After dialysis and neutralization with phosphate buffer the final vol. was made up to 20 ml. By the ninhydrin method

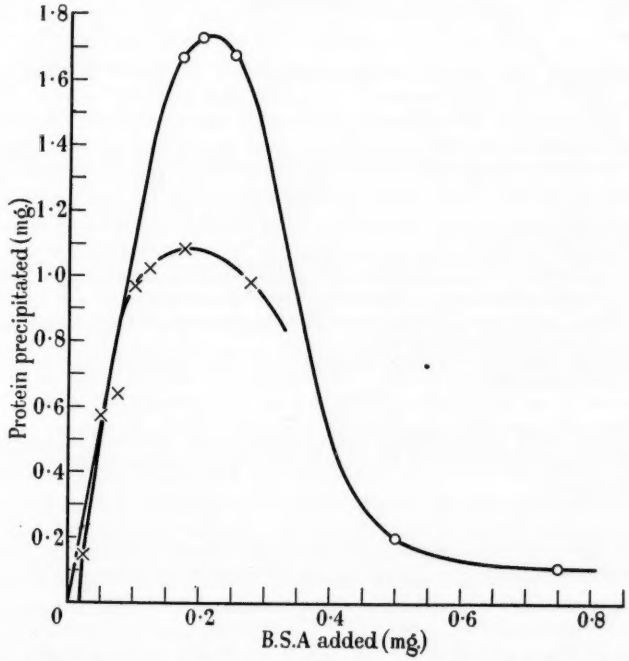

FIG. 3.—Precipitation curves. 0·5 ml. acetylated anti-B.S.A.-γ-globulin (58); varying amounts of B.S.A. added.

×—× Precipitates obtained with acetylated anti-B.S.A.-γ-globulin alone.

○—○ Precipitates obtained with mixtures of acetylated anti-B.S.A.-γ-globulin and antigen, to which 0·4 ml. anti-B.S.A. serum, B 1/4 (containing 0·280 mg. antibody) was added after 1 hr. Mixtures left at room temperature 1 hr. and at 4° for 3 days.

57 per cent of the NH_3^+ groups were found to be blocked. The apparent antibody estimated as described in *Methods* was 150 per cent of that calculated. The treated γ-globulin (0·5 ml.), without addition of untreated antiserum, formed a ppt. with H.S.A. (Table III). The maximum total ppt. (Table I, col. 4) formed with 0·45 mg. of H.S.A., was 5·0 mg. The 0·8 ml. of treated γ-globulin would have contained 1·76 mg. of antibody if its antibody content had remained unaltered by the treatment; the 0·4 ml. of antiserum 128 added (Table III) contained 2·39 mg. of antibody. The apparent antibody precipitated: 5·00 (total ppt.) —

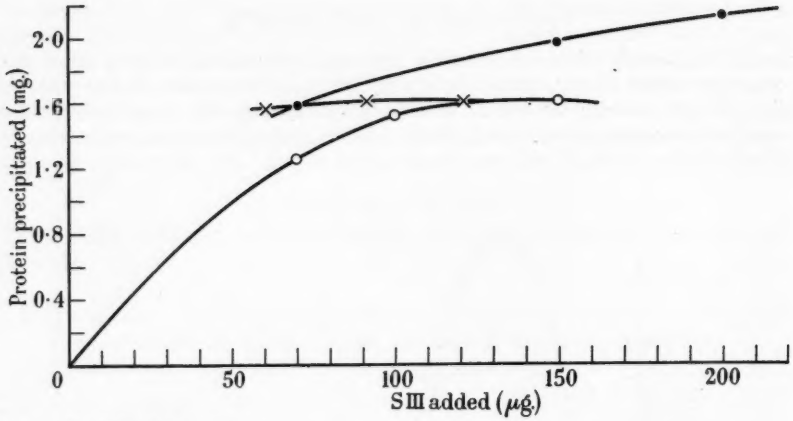

FIG. 4.—Precipitation curves:

●—● 0·5 ml. of acetylated globulin, with varying amounts of Type III polysaccharide; after 4 hr. at room temp. 0·1 ml. untreated globulin solution containing 0·47 mg. antibody was added. Mixtures left at 4° for 3 days.

○—○ 0·5 ml. acetylated globulin, with varying amounts of Type III polysaccharide. No untreated antibody added.

×—× 0·5 ml. untreated globulin, diluted 1/6·5, with varying amounts of Type III polysaccharide.

0·45 (antigen) = 4·55 mg. slightly exceeds the total amount of antibody :—1·76 + 2·39 = 4·15 mg. that would be present if that in the treated γ-globulin had remained unaltered.

TABLE III.—*Precipitates Formed by Mixtures of Treated Anti-horse Serum Albumin Gamma Globulin with Varying Amounts of Horse Serum Albumin.*

H.S.A. (mg.).	Ppt. I (mg.).	Ppt. II (mg.).	Ppt. I + Ppt. II (mg.).
0·09	0·73	0·79	1·52
0·18	0·98	0·93	1·91
0·27	1·18	1·76	2·94
0·45	0·76	4·24	5·00

Ppt. I: Amounts of precipitate formed by mixtures of 0·8 ml. of treated anti-H.S.A.-γ-globulin with varying amounts of H.S.A.

Ppt. II: Amounts of precipitate formed after removal of Ppt. I and addition to each of the supernatants of 0·4 ml. anti-H.S.A.-serum 128, containing 2·39 mg. of antibody.

As the amount of apparent antibody recovered from the treated γ-globulin exceeded the calculated antibody, some aggregation must have occurred. Although the presence of an excess of acetamide does reduce cross linking by formaldehyde, Fraenkel-Conrat and Mecham (1949) found some evidence of aggregation, as the apparent mol. wt. of ovalbumin was increased by this treatment.

This treated γ-globulin was examined by paper electrophoresis. Two distinct fractions appeared, both of which had higher mobilities than that of the original γ-globulin which was run simultaneously on the same paper; this increase of

mobility is expected, as the neutralization of NH_3^+ groups would give the molecule a higher overall negative charge. A component of the mixture of formaldehyde-acetamide-γ-globulin with B.S.A. remained at the origin. This was presumably the B.S.A.-antibody component; the antibody part seems to have been derived mainly from the slower component, as the area corresponding to this component was reduced more than the area corresponding to the faster component.

DISCUSSION.

Antigens.

The outstanding result of these experiments is that the acetylated protein antigens still formed precipitates with the corresponding antibodies. However, their reactions differed from those of untreated proteins in two respects. Flocculation was slower and the maximum amount of precipitate formed was less. Acetylated B.S.A. and ovalbumin precipitated about two-thirds of the corresponding antibody; Wetter and Deutsch (1950) found that about the same proportion of antibody was precipitated by acetylated ovomucoid from anti-ovomucoid serum. We cannot ascribe the formation of precipitates to the presence either of molecules that had entirely escaped acetylation or of molecules in which the hypothetical NH_3^+ groups of the receptors were unaffected, although other NH_3^+ groups of the molecule had been acetylated. For in either of these cases more of the acetylated than of the untreated antigen would have been required to precipitate a given amount of antibody, and when enough was added all the antibody would be precipitated. The precipitation curves would be elongated as if the scale of the abscissae were increased. Fig. 1 shows the similarity between the curve obtained with acetylated B.S.A. and that which would be obtained with untreated B.S.A. reacting with the same amount of antibody.

As only part of the antibody was precipitated by the acetylated antigen we must infer that the antibody molecules are not uniform. It might be suggested that, in the sera under consideration, this heterogeneity takes the form of the presence of two types of antibody—Type A, which combines only with determinants that contain NH_3^+ as essential parts, and Type B, which combines with determinants that do not contain NH_3^+. Two-thirds of the antibody would be of Type B and the remaining third of Type A. If this supposition were correct the acetylated antigen should not combine with Type A antibody.

However, as the rates of flocculation of B.S.A. and ovalbumin with the homologous antisera are much reduced when these antigens are acetylated, it is probable that the effect of acetylation is not confined to the destruction of certain determinants on these antigens and consequent inactivation of the corresponding antibodies which account for $\frac{1}{3}$ only of the total antibody. It is not necessary to postulate the presence of antibodies that combine only with NH_3^+-containing determinants. Instead of supposing that the heterogeneity of antibody takes the form of affinity for different determinants on the same antigen molecule, we may suppose that an antiserum contains a range of molecules that differ in affinity for the same determinant. Influences that reduce the probability of collision and combination of antigen and antibody molecules will inhibit the combination with those antibody molecules that have the least affinity for antigen.

According to Goldberg's (1952) formulation of the process of formation of precipitates by antigens and antibodies, anything that checks the process of

aggregation up to the actual " gel point " will inhibit formation of a precipitate. Antibody molecules compete with other ions and with water molecules for combination with antigen molecules ; the chance of collisions between antigen and antibody molecules and between antigen-antibody aggregates is reduced by repulsion due to like overall negative charges. An increase of affinity for other ions or a reduction of the specific affinity of antigen for antibody will reduce the probability that collisions will result in combination. Also, as antibody-antigen aggregates grow, the probability that a collision will bring free determinant and receptor into apposition becomes progressively less, whereas the overall charges are unaffected ; the effect of repulsion due to like charges increases with growth of the aggregates and aggregation may stop short of the gel point.

Acetylation of NH_3^+ groups reduces the probability of collision as it increases the overall negative charge of B.S.A. and ovalbumin. It is also possible that replacement of NH_3^+ groups by neutral groups leads to distortion of the molecules comparable to that produced in ovalbumin, B.S.A. and rabbit γ-globulin at high pH levels (Beaven and Holiday, 1952). Such distortion may affect the determinants of the antigens and reduce the antibody-antigen affinity. It is significant that the antigen least affected by acetylation was lysozyme (Table I, Fig. 2). For, owing to the large number of guanidinium groups in the molecule, lysozyme is positively charged between pH 7 and 8 (the range in which the reactions were studied) even when the NH_3^+ groups are acetylated. Also the lysozyme molecule is particularly stable ; it is remarkably resistant to denaturation (Fraenkel-Conrat, 1950) and the effects of alkali on it are fully reversible (Fromageot and Schnek, 1950) ; it is therefore less likely to be distorted when the NH_3^+ groups are acetylated.

Antibodies.

The acetylated globulins either flocculated slowly with the corresponding protein antigens, horse albumin, B.S.A., ovalbumin and diphtheria toxin or did not flocculate at all during the period of observation. After untreated antibody had been added to co-precipitate the acetylated incomplete antibody, flocculation was slow and the apparent antibody precipitated was less than the antibody in the solution before acetylation. The hypothesis that the globulin solutions contained two types of antibody—Type A in which NH_3^+ groups were essential parts of the receptors and Type B in which they were not—can be ruled out, for the Type B antibodies should flocculate normally with antigen even when acetylated. As with acetylated antigens, we may attribute the slow flocculation and incomplete precipitation of antibody to distortion of the antibody molecules and to the increase of negative charge. The rate of flocculation and completeness of precipitation should be more affected by increased negative charge of antibody than by increased charge of antigen because the antigen-antibody aggregates are mainly composed of antibody.

The precipitation curve of the mixture of acetylated antibody and untreated antibody falls steeply in antigen excess ; this recalls the curves found by Orlans (1953) with mixtures of lipid-extracted and untreated sera. The curve obtained with acetylated anti-B.S.A. 58 (Fig. 3) appears not to go through the origin ; *i.e.*, there was a prozone, such as is found with horse antiprotein sera. Goldberg and Williams (1953) consider that such a prozone is not found with rabbit antisera because of the relative insolubility of rabbit antibodies ; formation of a precipitate

by rabbit antibodies would be inhibited when antibody was in excess or antigen in relatively slight excess, if the solubility of the antibody was increased or formation of aggregates checked by some other cause.

Acetylation had a greater effect on digested diphtheria antitoxin than on the antibodies against serum albumin and ovalbumin. Sandor and Goldie (1938) found that the neutralizing titre of diphtheria antitoxin was considerably reduced when more than 50 per cent of the NH_3^+ groups were acetylated with ketene, although the tyrosine OH groups were not appreciably affected until 80 per cent of the NH_3^+ groups were acetylated. Katsura (1951) found that when digested antitoxin was acetylated with ketene, the neutralizing titre fell faster than the number of free NH_3^+ groups.

It might be expected that positively charged groups would be essential parts of the receptors of the two anti-pneumococcal antibodies, since the determinants of S III certainly, and of S I, probably, contain acid groups. Nevertheless, acetylated Type I and Type III antibodies formed precipitates with S I and S III, without the addition of untreated antibody. In fact, the reaction of the acetylated Type III antibody (rabbit) was exceptional, as it flocculated rapidly with S III and no more was co-precipitated with untreated antibody than was precipitated directly. However, the precipitation curves of acetylated and untreated antibodies differed (Fig. 4); more polysaccharide was needed to precipitate a given amount of antibody from the acetylated solutions. This is very like the result found by Chow and Goebel (1935) with Type I antibody, acetylated with ketene. These authors suggest that NH_3^+ groups are involved in the combination of antibody with Type I polysaccharide but that other reactive groups may be involved. These other groups may be the more strongly basic guanidinium groups. It is especially likely that guanidinium groups are involved in the combination of antibody with the Type III polysaccharide, which is relatively strongly acid. The positively charged guanidinium may, possibly, be involved in the combination of antibodies with protein antigens also. This possible involvement of guanidinium groups has not been studied.

One further possibility may be considered. Each determinant of a protein antigen may consist of a pattern of negatively charged COO^- groups and positively charged NH_3^+ groups which fit a complementary pattern of NH_3^+ and COO^- groups in the receptor of the antibody. When the NH_3^+ groups of either partner are blocked by acetylation the COO^- groups of this partner remain to attract the NH_3^+ groups of the non-acetylated partner. If both antigen and antibody are acetylated they should not combine. Acetylated B.S.A. does not form a precipitate with acetylated antibody; but Dr. Ottewill, from light scattering and ultracentrifuge measurements found that they do form complexes. It seems, therefore, that the NH_3^+ groups of protein antigens and of the corresponding antibodies are not essential for combination. This conclusion agrees with Pappenheimer's (1938) observation that acetylation of the NH_3^+ groups of diphtheria toxin did not affect its combination with antitoxin although it delayed flocculation. Heidelberger and Maurer (1952) removed 30 per cent of the NH_3^+ groups of ovalbumin with nitrous acid; the reaction with antibody was little affected by this treatment. On the other hand, Grabar and Kaminski (1950) found that ovalbumin, in which 75 per cent of the NH_2 groups had been acetylated with ketene, did not form a precipitate with antiserum. The effects with ketene and with acetic anhydride differ; it is possible that ketene acetylates the OH

groups of the tyrosine of ovalbumin before a large proportion of the NH_3^+ groups are acetylated.

As mentioned in the introduction, conclusions based on amounts of precipitate will be vitiated if the molecules of the protein antigens or of γ-globulins are aggregated after acetylation. Two of these—acetylated B.S.A. (Table I, Exp. 2) and acetylated anti-B.S.A. γ-globulin (Table II, Exp. 5)—were examined by Dr. Ottewill by a light scattering method and no evidence of aggregation was found as the molecular weights were 70,000 and 160,000 respectively; details of these studies will be published separately. Also, if either antigen or antibody were aggregated the precipitation curves should be abnormal; those found (*e.g.*, Fig. 1) are very similar to those given by untreated antigens and antibodies.

There was evidence that the antibody in the γ-globulin treated with formaldehyde and acetamide was aggregated although little over half the NH_2 groups were modified. This method is therefore not a satisfactory one for studying the effect of blocking NH_2 groups.

SUMMARY.

A large proportion of the amino groups of protein antigens and of antibodies were acetylated by treatment with acetic anhydride; the hydroxyl groups of tyrosine were not acetylated by this treatment.

Protein antigens in which a large proportion of the amino groups were acetylated formed precipitates with the corresponding antisera. Precipitation was delayed and the antibody was not completely precipitated; these changes were least with acetylated lysozyme.

Formation of precipitates by untreated antigens with acetylated antibodies (with the exception of rabbit antibody against Type III pneumococcus) was much delayed and in some cases did not occur at all during the period of observation.

Acetylated antibodies were partially carried down in the precipitates formed by the homologous antigens with untreated antibodies. It is inferred that they had been converted into incomplete antibodies.

Combination of protein antigens with the corresponding antibodies can occur when the amino groups of either or both are acetylated.

The delay or failure of precipitation is attributed to distortion of the molecules of acetylated proteins and increase of their negative charges.

It is suggested that an antiserum against Type III pneumococcus and, possibly, other antisera may contain two types of antibody and that amino groups are essential parts of the receptors of one of these types of antibody.

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INACTIVATION OF ANTIBODIES BY ULTRAVIOLET RADIATION.

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QUANTUM yields for inactivation of biologically active proteins, such as enzymes, by ultraviolet radiation of $\lambda = 2537$ Å may vary with environmental conditions such as pH of the medium. Therefore, any attempt to correlate the quantum yields with any other feature of the proteins necessarily involves a somewhat arbitrary choice of values from ranges of variation. Nevertheless the quantum yields (ϕ) tend to decrease with increasing molecular weights (M), and McLaren, Gentile, Kirk and Levin (1953) consider that the relationship between them is approximately expressed by the equation :

$$\phi = Q/M \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$

where Q is a constant for which they suggest the value of 447. The equation may have to be modified or abandoned as more data are obtained, but at present it fits reasonably well to the quantum yields of insulin, several enzymes and tobacco mosaic virus (McLaren, 1949 ; McLaren *et al.*, 1953). The work described below was done mainly to see whether the quantum yields for the inactivation of antibodies also approximately fit to equation (1).

Davis, Hollaender and Greenstein (1942) showed by electrophoresis that exposing sera to ultraviolet radiation causes the different protein fractions to combine and form complex aggregates. Before attempting to measure the quantum yields, therefore, it was first necessary to find whether irradiated but still active antibody and non-antibody proteins combine, and, if so, whether the resulting complexes influence results of tests for the residual activities of irradiated antibody preparations.

Hanan (1952), who studied a globulin fraction of antiserum to bovine serum albumin, found that exposure to ultraviolet radiation alters the behaviour of still active antibody, although he concluded that active antibody and non-antibody globulin did not combine. However, when he later irradiated whole antiserum, he obtained evidence for the production of complexes between still active antibody and non-antibody proteins (personal communication).

MATERIALS AND METHODS.

Antigens and antisera.—Tobacco mosaic virus (T.M.V.), tomato bushy stunt virus (B.S.V.) and a strain of clover nodule bacteria (Cl 15) were used as antigens. They and their antisera were prepared as previously described (Kleczkowski, 1950).

Water-soluble euglobulin.—This was obtained by adding one vol. of a saturated soln. of $(NH_4)_2\ SO_4$ to two vol. of a serum, separating the ppt. by centrifugation, washing it twice in

a 1/3 saturated soln. of $(NH_4)_2 SO_4$, dissolving in water, dialysing against water and removing by centrifugation the ppt. of insoluble euglobulin.

Albumin.—This was prepared by adding a saturated soln. of $(NH_4)_2 SO_4$ to an equal vol. of serum, removing the globulin ppt. by filtration, precipitating total albumin by complete saturation with $(NH_4)_2 SO_4$, dissolving the ppt. in water and dialysing against water.

The source of ultraviolet (u.v.) radiation.—This was a vitreosil low-pressure mercury lamp provided with a chromium-plated cylindrical reflector. Most of its total radiation was of a λ of 2537 Å. The intensity of radiation (measured with a thermopile and also with the apparatus described by Latarjet, Morenne and Berger, 1953) at a distance of 10 cm. from the lamp was about 3000 μw/sq. cm.

Absorption of the u.v. radiation of λ 2537 Å *by protein solutions.*—This was measured with a quartz spectrophotometer.

Irradiation.—Petri dishes containing layers of solutions 0·14 cm. deep were placed at 10 cm. from the lamp and rocked continuously during irradiation ; this is assumed to have ensured equal exposure of all molecules. Any loss of water by evaporation during irradiation was made up by adding water.

Serological titrations.—One ml. samples of increasingly diluted antibody preparations were added to each of a series of tubes containing 1 ml. of antigen at a constant concn., 0·005 per cent for each of the two viruses and 10^9 cells per ml. for the bacteria. The tubes were placed in a waterbath at 50°, with the fluid columns partly immersed to cause convection. The appearance and degree of flocculation were observed at intervals during 3 hr.

Antibody preparations that failed to flocculate their antigens were tested for their ability to inhibit flocculation ; 0·1 ml. of suitably diluted untreated antiserum was added to each mixture that failed to flocculate after 3 hr. incubation at 50°. Continued absence of flocculation was taken as evidence of inhibition.

RESULTS.

Irradiation of whole antisera.—Table I shows that the flocculation titres of all the three antisera decreased progressively with increasing time of irradiation, but that they differed considerably in their response to irradiation. When irradiated at a diln. of 1/50, the flocculation titres of the two virus antisera decreased at about the same rate, whereas that of the bacterial antiserum decreased about four times as fast. Changing the diln. at which the antisera were irradiated from 1/50 to 1/10 gave different results with each of the three antisera. The differences between the two virus antisera were quantitative. Irradiating at 1/10 instead of 1/50 increased the rate at which the flocculation titre decreased and it introduced specific inhibition, but both effects were much more pronounced with B.S.V. than with T.M.V. antiserum. The bacterial antiserum behaved qualitatively differently from the virus antisera. The change of diln. slowed the rate at which the flocculation titre decreased and did not develop the ability to inhibit.

The results obtained by irradiating antisera to the two viruses resemble those obtained by heating them (Kleczkowski, 1941*c* ; 1950) ; they could be explained by assuming that, at a diln. of 1/10 but not at 1/50, antibodies combine with other proteins to form non-flocculating and inhibiting complexes. The differences between the behaviour of the two antisera can be explained by assuming that precipitation of T.M.V. is less readily inhibited by non-precipitating complexes than is precipitation of B.S.V., which is true for complexes formed by heat. The results obtained by irradiating the bacterial antiserum, however, differed strikingly from those obtained by heating and could not be explained on previous knowledge. To explain them it was necessary to irradiate globulin fractions of antisera alone and with added albumin.

TABLE I.—*Effects of Ultraviolet Irradiation of Whole Antisera.*

Dilutions at which antisera were irradiated.	Time of irradiation (min.).	Serological test: Dilutions of antisera. 1 in— 12·5.	25.	50.	100.	200.	400.	800.	1600.	3200.	6400.	12,800.
Antiserum to B.S.V.												
	0	+++	+++	+++	+++	+++	++	+	−	.	.	.
1/50	15	.	.	+++	+++	++	+	−	−	.	.	.
	30	.	.	+++	++	+	−	−	−	.	.	.
	60	.	.	+	−	−	−	−	−	.	.	.
1/10	15	+	+	++	++	+++	+	−	−	.	.	.
	30	i	i	i	i	o	o	o	o	.	.	.
	60	i	i	i	i	i	o	o	o	.	.	.
Antiserum to T.M.V.												
	0	+++	+++	+++	+++	+++	+++	++	+	−	.	.
1/50	15	.	.	+++	+++	+++	++	+	−	−	.	.
	30	.	.	+++	+++	++	+	−	−	−	.	.
	60	.	.	++	+	−	−	−	−	−	.	.
1/10	15	+++	+++	+++	+++	++	+	±	−	−	.	.
	30	i	+	++	++	+	−	−	−	−	.	.
	60	i	i	o	o	o	o	o	o	o	.	.
Antiserum to Cl 5 bacteria:												
	0	.	.	.	.	+++	+++	+++	+++	++	+	−
1/50	7·5	.	.	.	.	+++	+++	++	+	−	−	−
	15	.	.	.	.	++	+	−	−	−	−	−
	30	.	.	.	.	−	−	−	−	−	−	−
1/10	7·5	.	.	.	.	+++	+++	+++	++	+	−	−
	15	.	.	.	.	+++	+++	++	+	−	−	−
	30	.	.	.	.	++	+	−	−	−	−	−

+ = the presence and degree of flocculation.
− = the absence of flocculation when the test for inhibition was not made.
i = the presence of inhibition.
o = the absence of inhibition.
. = test not made.

The inhibition tests were made by adding 0·1 ml. of untreated antiserum at a dilution of 1/10 to the tubes where there was no flocculation after 3 hr. incubation.

Complex formation between euglobulin and albumin.—The ability of u.v. radiation to form complexes between euglobulin and albumin was examined by the method previously used to examine complexes formed in heated mixtures (Kleczkowski, 1941*b*; 1950) and based on precipitation with salt.

Three solutions containing:

A. 0·2 per cent of the euglobulin,
B. 0·2 per cent of the euglobulin and 1 per cent of the albumin,
C. 1 per cent of the albumin,

all in 0·067 M phosphate buffer at pH 6·8, were irradiated. The periods of irradiation were so adjusted that the euglobulin in soln. A absorbed as much radiation energy as did the euglobulin in soln. B, and so that the albumin in soln. C absorbed as much as did the albumin in solution B.

The proportion (p) of incident radiation energy absorbed by a soln. of a protein is

$$p = 1 - \exp\ (-2{\cdot}3\ \alpha c x), \qquad (2)$$

where α is the extinction coefficient of the protein, c its concentration in mg./ml. and x the thickness of the layer of the irradiated solution in cm. When a mixture of two different proteins is irradiated, the proportion of incident radiation energy absorbed by the solution is :

$$p = 1 - \exp\ [-2{\cdot}3\ (\alpha_1 c_1 + \alpha_2 c_2)\ x], \qquad (3)$$

where α_1, and c_1 are the extinction coefficient and the concn. of one protein and α_2 and c_2 are those of the other. The ratio of the proportion (p_1) of the radiation energy absorbed by one protein component of the mixture to that absorbed by the other (p_2) is

$$p_1 / p_2 = \alpha_1 c_1 / \alpha_2 c_2. \qquad (4)$$

The extinction coefficients of the euglobulin and of the albumin, measured with a quartz spectrophotometer, were 0·72 and 0·36, respectively. They were assumed to remain constant during the irradiations, and absorption by the buffer, which was negligibly small, was disregarded.

From equations (2), (3) and (4), and the fact that the layer of the irradiated soln. was 0·14 cm. deep, to ensure that each protein fraction always absorbed the same amount of radiation energy, it was calculated that soln. B and C needed to be exposed respectively 1·65 and 1·365 times as long as soln. A.

To see whether euglobulin and albumin form complex aggregates, the irradiated soln. were mixed with equal vol. of 0.067 M phosphate buffer at pH 6·8 or with equal vol. of other irradiated or non-irradiated soln. A saturated soln. of $(NH_4)_2\ SO_4$ was then added to the mixtures in an amount sufficient to precipitate the euglobulin alone and a proportion of the euglobulin-albumin complex (if any were formed) but not albumin alone. The ppt. were sedimented by centrifugation at 8000 r.p.m., washed 3 times in a 5 per cent solution of trichloroacetic acid, and their protein contents determined (Micro-Kjeldahl).

Table II shows that $(NH_4)_2\ SO_4$, at concn. insufficient to precipitate irradiated albumin alone (Test no. 2) but sufficient to precipitate the euglobulin (Test no. 1), precipitated some of the albumin together with the euglobulin from mixtures of the two (Tests no. 3 and 4). More albumin was precipitated if the two proteins were first mixed and then irradiated (Test no. 4) than *vice versa* (Test no. 3). Non-irradiated albumin was not precipitated with the irradiated euglobulin (Test no. 5).

There are three reasons for considering that the albumin precipitated because it was combined with the euglobulin. Firstly, the amounts precipitated seem too great to occur by adsorption on the ppt. of irradiated euglobulin. Secondly, no non-irradiated albumin precipitated with irradiated euglobulin showing that the ppt. of irradiated euglobulin did not tend to adsorb the other protein. Thirdly, more of irradiated albumin precipitated in Test no. 4 than in Test no. 3. The conclusion seems inescapable that some of the albumin combined with the euglobulin when the two were irradiated together, and it seems probable that some,

TABLE II.—*Precipitability of Euglobulin and Albumin by Ammonium Sulphate from Irradiated Solutions.*

Solution.	Contents of solutions (in g./l). Euglobulin.	Albumin.	Time of irradiation (min.).	Radiation energy (in joules) absorbed per mg. of— Euglobulin.	Albumin.
A	2	..	30	7·16	..
B	..	10	40·5	..	3·58
C	2	10	48·5	7·16	3·58
D	..	10	Non-irradiated		

All the solutions were in 0·067 M phosphate buffer at pH 6·8.

Precipitation with ammonium sulphate.

Test No.	Composition of mixtures (in ml.). A. Irrad. euglob.	B. Irrad. alb.	C. Irrad. mixture.	D. Non-irrad. alb.	Buffer.	Mg. of protein precipitated by adding saturated solution of $(NH_4)_2SO_4$. 2 ml. (33 per cent saturated).	2·5 ml. (38·5 per cent saturated).
1	2	..	..	..	2	3·6	3·6
2	..	2	..	..	2	0	2·4
3	2	2	..	..	..	5·9	8·0
4	..	..	2	..	2	6·7	11·7
5	2	..	..	2	..	3·5	..

though less, also combined when the two were mixed after being irradiated separately. This would be a distinguishing feature between results of irradiation and of heating, as proteins mixed after they have been heated separately do not seem to form complexes (Kleczkowski, 1941*b*).

The result of irradiating the mixtures of the two proteins (Table II, Test no. 4) is directly comparable with the result of heating a similar mixture (Kleczkowski, 1950, Table III, Test no. 3), for in both experiments the periods of treatment were such that antibody to B.S.V. would have lost its ability to precipitate the virus and developed the ability to inhibit its precipitation ; the protein concn., the pH and the buffer used were the same in both experiments. If, by analogy with the experiment with the heated proteins, it is assumed that most of the euglobulin was precipitated by $(NH_4)_2SO_4$ from the irradiated mixture in Test no. 4 (Table II), and that all or most of the albumin that was precipitated with it was combined with it, irradiation seems to cause less combination than is caused by heating.

Irradiation of Antibodies in Isolated Euglobulin Fractions with and without Addition of Albumin.

The euglobulin fraction of each of the three antisera was irradiated at a concn. of 0·04 per cent alone and in the presence of 0·4 per cent albumin. Non-irradiated soln. of the euglobulin and those irradiated alone were also mixed with equal volumes of a 0·4 per cent soln. of the albumin that had been irradiated separately. All the soln. contained 1 per cent NaCl and were at pH 7. The ratios of times of

irradiation of different soln. were computed as described above (using equations (2), (3) and (4)) to ensure that each of the two proteins absorbed the same amount of radiation energy when irradiated alone as it did when irradiated with the other.

Serological tests of the resulting preparations (Table III) showed alterations in behaviour of antibodies to B.S.V. and to the bacteria, resulting from their combination into a complex with albumin when irradiated together with it. With antibody to BSV this resulted in the loss of the ability to flocculate the virus and in acquiring the ability to inhibit its flocculation by unchanged antibody. By contrast, when the antibody to bacteria was combined with albumin the rate of loss of its ability to flocculate the antigen was decreased.

Comparison of these results with those recorded in Table I shows that the euglobulins, when irradiated alone, behaved like whole antisera irradiated at a diln. of 1/50, and, when irradiated in the presence of albumin, they behaved like whole antisera irradiated at a diln. of 1/10. It is concluded, therefore, that when whole antisera were irradiated at 1/10, antibodies formed complexes with other protein fractions (particularly with albumin), and that this was prevented at 1/50.

In contrast to the complex between B.S.V. antibody and albumin, the complex between the irradiated bacterial antibody and albumin behaved quite differently from those formed by heat (cf. Kleczkowski, 1941*c*; 1950); instead of failing to flocculate and inhibiting, it gave higher flocculation titres than antibody irradiated without added albumin. It is possible that combination with albumin protects the antibody from damaging effects of the radiation. This does not seem likely, however, because the other antibodies were not so protected. Another possibility is that irradiated albumin interacts with the bacterial surface making the suspension less stable, whereas heated albumin does not. This is supported by the fact that adding irradiated albumin to either irradiated or non-irradiated antibody preparations slightly increased their titres (see Table III). This was not observed with heated albumin preparations.

If complexes are formed between separately irradiated and then mixed antibodies and the albumin, as suggested by the results recorded in Table II, they are not formed on a scale sufficient to affect the behaviour of antibody preparations, although the slight drop in the titre of irradiated B.S.V. antibody to which albumin, which had been irradiated separately, had been added (cf. Tests 4 and 5, Table III) could have been caused by the formation of such a complex.

The behaviour of antibody to T.M.V. remained unaltered by the presence of albumin during irradiation. With this antigen, therefore, adding albumin did not reproduce the effect of changing the diln. at which the whole serum was irradiated from 1/50 to 1/10 (cf. Table I). This can be explained by assuming that precipitation of T.M.V. by its antibody is less affected by complex formation between the antibody and albumin than is precipitation of B.S.V., which is true for complexes formed by heat (Kleczkowski, 1941*c*). This assumption would also explain the much smaller effect of changing the diln. at which whole antiserum to T.M.V. was irradiated from 1/50 to 1/10, than was obtained with whole antiserum to B.S.V. (Table I). It is concluded, therefore, that in the conditions of the experiment recorded in Table III, complex formation had probably occurred on a scale insufficient to affect precipitation of T.M.V., whereas irradiation of the whole antiserum at a dilution of 1/10 resulted in complex formation on a scale just large enough to make the antibody unable to precipitate the virus and able to inhibit its precipitation.

The Rates of Inactivation of Antibodies and the Quantum Yields.

The rates of inactivation of antibodies by u.v. radiation were measured by the decrease in precipitin or agglutinin titres of irradiated soln. The euglobulin fractions of antisera were used and it was assumed that antibody and non-antibody euglobulin have the same extinction coefficient, about 0·72, and the same molecular weight, about 170,000 (Svedberg, 1937).

As no serologically detectable complex formation occurred between antibody and albumin when whole antisera were irradiated at a diln. of 1/50, *i.e.*, when the total protein concentration was slightly over 0·1 per cent, it was assumed that, when the euglobulin fractions were irradiated at a concn. of 0·02 per cent, antibody molecules did not combine with each other or with non-antibody euglobulin.

A few preliminary experiments determined the times of irradiation during which the titre of each euglobulin decreased to half. Table IV shows that this was 6·9 min. for the euglobulins of the two virus antisera and 2·8 min. for the euglobulin of the bacterial antiserum, and that the course of inactivation of all the antibodies approximated that of a first order reaction, at least until the residual activity fell to about 10 per cent of the original. The proportion (r) of activity remaining after the time t (in min.) of irradiation can thus be considered as following the equation $r = e^{-kt}$, where k is a constant the value of which is about 0·1 for the antibodies to the two viruses and about 0·25 for the antibody to the bacteria.

The irradiated solutions did not inhibit the flocculation of their antigens by non-irradiated antibodies.

If it is assumed that the extinction coefficient did not change appreciably during irradiation, the quantum yields (ϕ) can be obtained by substituting the value of the constants k into the equation :

$$\phi = km \,/\, Ep, \qquad . \quad . \quad . \quad . \quad . \quad (5)$$

where m is the amount of euglobulin in moles under each sq. cm. of irradiated area, which was $1{\cdot}65 \times 10^{-10}$ (the thickness of the irradiated layer was 0·14 cm.); E is the amount of radiation energy in einsteins falling during each minute on each sq. cm. of irradiated area, which was $3{\cdot}8 \times 10^{-7}$ (3000 μw per sq. cm., the wave-length being 2537 Å) and p is the proportion of incident radiation absorbed by the euglobulin, obtained from equation (2), and was 0·0454. Substituting these values into equation (5) the quantum yield of about $0{\cdot}96 \times 10^{-3}$ is obtained for the antibodies to the two viruses and about $2{\cdot}4 \times 10^{-3}$ for the antibody to the bacteria.

Equation (1) gives the value of $2{\cdot}6 \times 10^{-3}$ for the quantum yield expected for a protein whose molecular weight is 170,000. This value is close to that obtained for the antibody to the bacteria and slightly more than twice that obtained for the antibodies to the two viruses. It can be concluded, therefore, that the quantum yields of antibodies fall into line with those of other specifically active proteins.

DISCUSSION.

The approximate inverse proportionality between quantum yields and molecular weights means that the extent of inactivation is a function of the amount of

TABLE III.—*Effects of Irradiation of Euglobulin Fractions of Antisera Alone and in the Presence of Serum Albumin.*

Test No.	Contents of irradiated solutions (in g./l.). Euglobulin.	Albumin.	Time of irradiation (min.).	Radiation energy (in joules) absorbed per mg. of Euglobulin.	Albumin.	Serological test. The dilutions of 0·04 per cent euglobulins. 1 in— 1.	2.	4.	8.	16.	32.	64.	128.	256.	512.	1024.
						Euglobulin from Antiserum to B.S.V.										
1	0·4	0	0	0	—	+++	+++	+++	+++	++	+	−	.	.	.	.
2	0·4	0	7·0	2·0	—	+++	+++	+++	++	+	−	−	.	.	.	.
3	0·4	4	8·7	2·0	1·0	+++	+++	+++	++	+	−	−	.	.	.	.
4	0·4	0	23·5	6·7	—	+++	++	+	−	−	−	−	.	.	.	.
5	0·4	0	23·5	6·7	—	.	+	−	−	−	−	−	.	.	.	.
	0	4	28·1	—	3·35 *m*											
6	0·4	4	29·3	6·7	3·35	i	i	i	o	o	o	o	.	.	.	.
7	0·4	0	0	0	—	+++	+++	+++	+++	++	+	−	.	.	.	.
	0	4	28·1	—	3·35 *m*											
						Euglobulin from Antiserum to T.M.V.										
8	0·4	0	0	0	—	+++	+++	+++	+++	++	+	−	.	.	.	.
9	0·4	0	23·5	6·7	—	+++	++	+	−	−	−	−	.	.	.	.
10	0·4	0	23·5	6·7	—	.	++	+	−	−	−	−	.	.	.	.
	0	4	28·1	—	3·35 *m*											
11	0·4	4	29·3	6·7	3·35	+++	++	+	−	−	−	−	.	.	.	.
						Euglobulin from Antiserum to Cl 5 bacteria.										
12	0·4	0	0	0	—	.	.	.	+++	+++	+++	+++	++	+	−	−
13	0·4	0	12	3·4	—	+++	+++	+++	++	+	−	−	.	.	.	.
14	0·4	4	14·95	3·4	1·7	+++	+++	+++	+++	++	+	−	.	.	.	.
15	0·4	0	20	5·7	—	++	+	−	−	−	−	−	.	.	.	.
16	0·4	0	20	5·7	—	.	+	±	−	−	−	−	.	.	.	.
	0	4	23·9	—	2·85 *m*											
17	0·4	4	24·9	5·7	2·85	+++	+++	++	+	−	−	−	.	.	.	.
18	0·4	0	0	0	—	.	.	.	+++	+++	+++	+++	+++	++	+	−
	0	4	23·9	—	2·85 *m*											

m indicates that the solutions were mixed in equal volumes after they had been irradiated separately.
All the other symbols and the method of testing for inhibition are the same as those in Table I.

TABLE IV.—*The Rate of Inactivation of Antibody.*

The euglobulin solutions were irradiated at a concentration of 0·02 per cent in 0·9 per cent NaCl at a pH 7·0.

Time of irradiation (min.).	Radiation energy (in joules) absorbed per mg. of euglobulin.	Serological test. Dilutions of the euglobulin solutions. 1 in— 2.	2·8.	4.	5·6.	8.	11·2.	16.	22·4.	32.	44·8.	64.	89·6.	128.	179·2.
Euglobulin of antiserum to B.S.V.															
0	0	+++	+++	+++	+++	+++	++	+	−	−	.	.	.	.	.
6·9	2·0	+++	+++	+++	++	+	−	−	−	−	.	.	.	.	.
13·8	4·0	+++	++	+	−	−	−	−	−	−	.	.	.	.	.
20·7	6·0	+	−	−	−	−	−	−	−	−	.	.	.	.	.
Euglobulin of antiserum to T.M.V.															
0	0	.	.	.	+++	+++	+++	++	+	−	.	.	.	.	.
6·9	2·0	+++	+++	+++	+++	++	+	−	−	−	.	.	.	.	.
13·8	4·0	+++	+++	++	+	−	−	−	−	−	.	.	.	.	.
20.7	6·0	++	+	−	−	−	−	−	−	−	.	.	.	.	.
Euglobulin of antiserum to Cl 5 bacteria.															
0	0	.	.	.	.	.	.	.	.	+++	+++	+++	++	+	−
2·8	0·8	.	.	.	.	+++	+++	+++	+++	+++	++	+	−	−	−
5·6	1·6	.	.	.	.	+++	+++	+++	++	+	−	−	−	−	−
8·4	2·4	.	.	.	.	+++	++	+	−	−	−	−	−	−	−

The symbols are the same as those in Table I.

radiation energy absorbed by a unit weight of a protein and is independent of the size of the molecules or particles making up the total mass. For example, the amount of energy of the radiation of λ 2537 Å, which must be absorbed by an enzyme or an antibody to reduce its activity to a half, is on average about 0·8 joule per mg., and individual deviations do not seem to exceed a factor of 4. Any conclusions from this must necessarily be postponed until enough information is obtained about the nature of active centres and their numbers per molecule.

The inverse proportionality is interpreted by McLaren *et al.* (1953) as indicating that the probability of " hitting " (*i.e.*, absorption by) the active centre of a molecule with a quantum decreases as molecular weight increases. This would imply that absorption of any number of quanta by any part of the molecule other than the active centre does not affect the activity of the molecule. It is also tacitly assumed that quantum yields, as they are usually obtained, are mean numbers of molecules inactivated by one quantum of radiation, and this would be so if each molecule had only one active centre or, if it had several, they were inactivated simultaneously, or if the whole of the molecule behaved as one active centre. If it had several active centres inactivated independently of one another, the quantum yield would be the mean number of active centres, and not of molecules, inactivated by one quantum of radiation absorbed by the protein.

Active centres of molecules of antibodies would, presumably, be the sites that combine specifically with antigens, and existing evidence suggests that there are two such sites per molecule (Pappenheimer, Lundgreen and Williams, 1940 ; Eisen and Karush, 1949 ; Marrack, Hoch and Johns, 1951). If so, there can be two possible mechanisms of inactivation, with no evidence to show which is more likely. The first is that inactivation of only one site of a molecule makes it unable to flocculate its antigen. As no inhibitory material was detected in irradiated preparations of the euglobulin fractions of antisera, this would imply that the " univalent " antibody molecules do not interfere with the activity of " bivalent " molecules, and inactivation of antibody proceeded as if both combining sites had been inactivated simultaneously.

The other possibility is that antibody molecules with one of their two combining sites inactive can still flocculate their antigen. This can be reconciled with inactivation following the course of a first order reaction only by assuming that the residual activity of an irradiated preparation is roughly proportional to the total concentration of the combining sites still remaining active. A number of molecules each with only one site still remaining active, would then be approximately equivalent to half the number of the originally " bivalent " molecules in their interaction with antigen. This would increase, though not more than double, the ratio of the weight of antibody to the weight of antigen at equivalence. The systems used in this work are not suitable for following changes in the antibody/antigen ratios, for these ratios are inconveniently low (see Kleczkowski, 1941*a*), but Hanan (1952) found that the ratio did increase progressively when a globulin fraction of antiserum to bovine serum albumin was irradiated. However, as the ratio increased much beyond twice the original value, some cause other than acquired " univalence " of antibody molecules must have been mainly or entirely responsible for the increase. This cause could have been complexes formed between antibody and non-antibody globulin, for the globulin fraction was irradiated at a sufficient concentration for complex formation to be expected.

As Hanan concluded that no such complexes were formed, some of his results

call for discussion. He found that, as the amount of irradiation increased, the globulin fraction showed equivalence with progressively decreasing amount of antigen, though the amount of precipitated antibody remained apparently almost unaltered so that the antibody/antigen ratio at equivalence increased and the total amount of precipitate decreased. The amount of precipitated antibody finally started falling progressively when the antibody/antigen ratio exceeded five times the original value.

The most likely explanation of his results seems to be that, as irradiation progressed and more antibody was inactivated, it became equivalent to less antigen; simultaneously active antibody was combining increasingly with non-antibody globulin in amounts that for some time happened to coincide approximately with the amount of antibody rendered inactive, hence the apparently almost unchanged amount of precipitated antibody. Hanan considered this explanation but rejected it because he thought it unlikely that the complex formation would balance the loss of active antibody and because he considered that the difference between results of irradiating and heating his globulin solution made a complex formation unlikely. He found that after heating for 15 min. at 75° the solution showed equilibrium with the same amount of antigen as originally, but the total amount of precipitate increased considerably showing that complex formation between antibody and non-antibody globulin had occurred.

The differences between Hanan's results with irradiated and heated globulin solution can be explained by differences in the relative rates of complex formation and of inactivation of antibody by heating and by radiation. During heating, much combination into complexes can occur before antibody is detectably inactivated, whereas irradiation inactivates much antibody before much combination into complexes can occur. For example, B.S.V. antibody heated with albumin combines with enough albumin to change from a precipitating to an inhibiting form before any measurable proportion of it is inactivated (Kleczkowski, 1950, Table II). By contrast, irradiated B.S.V. antibody does not combine with enough albumin to change its ability to precipitate into ability to inhibit until 75–90 per cent of its activity is destroyed (Table III).

SUMMARY.

When sufficiently concentrated mixtures of different serum proteins are exposed to ultraviolet radiation, complexes, *i.e.*, mixed aggregates composed of molecules of different proteins, are formed. Such complexes can also be formed between irradiated, but still active, antibodies and other serum proteins. The behaviour in flocculation tests of complexes formed between antibodies and an excess of serum albumin depends on the character of the antigen.

Inactivation of antibodies by ultraviolet radiation follows the course of a first order reaction. The quantum yields for inactivation by the radiation of λ 2537 Å are *c.* $2{\cdot}4 \times 10^{-3}$ for the antibody to clover nodule bacteria and *c.* $0{\cdot}96 \times 10^{-3}$ for the antibodies to tomato bushy stunt and tobacco mosaic viruses.

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